Analysis of porcine cytokine response upon *Streptococcus suis* infection

***in vivo,* in whole blood and in cell culture**

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Meinen Eltern und Großeltern

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INHALT

BIBLIOGRAPHISCHE DARSTELLUNG

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Analysis of porcine cytokine response upon *Streptococcus suis* infection *in vivo*, in whole blood and in cell culture

Fakultät für Lebenswissenschaften

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Dissertation

72 Seiten, 149 Literaturangaben, 8 Abbildungen, 5 Tabellen

Streptococcus (S.) suis ist ein weit verbreiteter Kommensale im Schwein, verursacht jedoch auch häufig vor Allem bei jungen Ferkeln invasive Infektionen, die sich in Septikämie und Meningitis äußern und teils gravierende Ausfälle in der Schweineaufzucht nach sich ziehen. Die zellulären Immunmechanismen, die dem Verlauf einer *S. suis*-induzierten Septikämie zugrunde liegen, sind kaum verstanden und wurden in der vorliegenden Studie durch Etablierung eines *in vitro*-Modells mit Primärzellen des Schweins anhand der Expression von typischen pro- (IL-6, TNF-α) und anti-inflammatorischen Zytokinen (IL-10) näher charakterisiert. Im Vergleich mit *in vivo*-infizierten Schweinen konnte gezeigt werden, dass mononukleäre Zellen des peripheren Bluts (PBMCs) nach Infektion mit *S. suis* vergleichbare Mengen IL-6 und IL-10 in einer ähnlichen engen zeitlichen Abfolge produzieren. *In vitro* wurde zusätzlich die Produktion von TNF-α nachgewiesen. Durch Versuche mit Kapselmutanten und verschiedenen Dosen von *S. suis* konnte gezeigt werden, dass die produzierte Zytokinmenge von der vorliegenden bakteriellen Last abhängt. IL-10 zeigte einen schwachen hemmenden Einfluss auf IL-6 und TNF-α. Mithilfe von Zellseparationsversuchen konnte weiterhin gezeigt werden, dass nach Infektion mit *S. suis* TNF-α hauptsächlich von Monozyten, nicht von Lymphozyten oder Granulozyten produziert wird. Die Ergebnisse ließen keine Schlüsse auf die zellulären Quellen von IL-6 und IL-10 zu. Abschließend wurde der Einfluss von TNF-α auf das Abtöten von *S. suis* in Abhängigkeit von der Konzentration spezifischer Antikörper gegen *S. suis* im Blut untersucht. Unabhängig von *S. suis*-spezifischem Immunglobulin (Ig) G hatte weder die Zugabe von rekombinantem TNF-α noch die Neutralisation von endogenem TNF-a eine Auswirkung auf das Abtöten von *S. suis*. Die etablierten *In vitro*-Modelle sind bezüglich Zytokinmenge und -kinetik vergleichbar mit den *in vivo*-Daten und liefern neue Erkenntnisse zur Zytokininduktion durch *S. suis,* zur Regulation der Zytokine und zu den zellulären Produzenten.

ABBREVIATIONS

°C	Degree Celsius
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDS	Colostrum-deprived serum
cfu	Colony-forming unit
cps	Capsular polysaccharide
DC	Dendritic cell
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EF	Extracellular factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FCM	Flow cytometric measurement
$x\,g$	Gravity
H_3PO_4	Phosphoric acid
IFN	Interferon
IMDM	Iscove's Modified Dulbecco's Medium
Ig	Immunoglobulin
IL	Interleukin
i.v.	intravenous
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MACS	Magnetic cell sorting
MLST	Multilocus sequence typing
MRP	Muramidase-released protein
MRSA	Methicillin-resistant *Staphylococcus aureus*
NET	Neutrophil extracellular trap
OD	Optical density
PAMP	Pattern-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PMA	Phorbol-12-myristat-13-acetat
PRR	Pattern recognition receptor
RT	Room temperature
SLY	Suilysin
SNP	Single nucleotide polymorphism
STSS	Streptococcal toxic shock syndrome
Th	T helper cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
WT	Wild type

1. INTRODUCTION

Streptococci are one of the most prominent groups of bacteria and can be found as commensals and pathogens across many vertebrate hosts, including humans. Until the beginning of the 20th century scarlet fever was one of the most problematic streptococcal infections for western societies. Even today, only four streptococcal species are responsible for a range of common human diseases: *Streptococcus* (*S.*) *pyogenes* (Lancefield group A streptococcus; e.g. pharyngitis, toxic shock syndrome, acute rheumatic fever), *S. agalactiae* (Lancefield group B streptococcus; e.g. sepsis in newborns), *S. pneumoniae* ("pneumococcus"; e.g. pneumonia, otitis media, meningitis, bacteremia), and *S. mutans* (e.g. dental caries) (*1*). Additional streptococcal species are common pathogens in domestic animals, including horses (*S. equi*, *S. zooepidemicus*, *S. pneumoniae*), cattle (*S. uberis*, *S. agalactiae*, *S. dysgalactiae*, *S. zooepidemicus*), dogs (*S. canis*), and pigs (*S. suis*, *S. porcinus*), some of which also have zoonotic potential (*2*). Since *S. suis* is being found in almost 100% of pig farms worldwide (*3*) and common serotypes in pig breeding have even been isolated form wild boars (*4*), *S. suis* is one of the most prevalent pathogens in pigs.

Since pigs are important for nutrition of many societies globally, *S. suis* outbreaks do not only happen frequently in pig herds, but occur also in humans, mostly causing meningitis (*5, 3*) and in some cases even Streptococcal toxic shock syndrome (STSS) (*6*). Most cases in humans occur in abattoir workers or people who are frequently exposed to raw meat (*5, 7, 8*). However, *S. suis* is among the leading causes of meningitis in Vietnam, Thailand and Hong-Kong (*9–12*) and infected 215 people in an epidemic outbreak among farmers in Sichuan Province, China, in 2005 with a mortality rate of 20%, and *S. suis* is consequently being recognized as an emerging zoonotic agent (*13, 14*).

The economic importance of pig breeding coupled with the epidemic potential in humans could be the reason for the increased research interest in *S. suis*, which is reflected by growing numbers of *S. suis*-related publications (*13*). Extensive research on the molecular characteristics and pathogen-host interactions of *S. suis* has been done using porcine, rodent and human models. However, the knowledge of the pathogenesis of *S. suis*-related disease is still limited and a cross-protective vaccine for pigs against multiple serotypes is still missing in Europe. Current research aims to expand the understanding of the pathogen and the development of a vaccine by combining expertise from both the human and the porcine background, which should ultimately benefit human and veterinary medicine alike.

1.1. *Streptococcus suis*

Streptococcus suis is a gram-positive, encapsulated coccus. *S. suis* expresses several molecular factors which are believed to be linked to virulence, some of the most investigated being suilysin

(SLY), muramidase-released protein (MRP), extracellular factor (EF), and the bacterial capsular polysaccharide (cps), the latter being used to discriminate between serotypes of *S. suis*.

Identification of *S. suis* serotypes

For diagnostic purposes, *S. suis* is cultivated on sheep blood agar plates where it typically forms gray-white colonies and shows α-hemolysis (incomplete hemolysis, i.e. oxidization of hemoglobin by hydrogen peroxide produced by the bacteria).

Serological characterization is based on the presence of different epitopes on the surface of the bacterial capsule. These epitopes can be detected by antibodies from antisera raised against reference strains. All currently established serological tests are based on the immunoreactivity of the reference sera with the capsular epitopes. The most common method is the co-agglutination test (*15, 16*); an inconclusive co-agglutination test can be followed by capillary precipitation or Neufeld's capsular reaction (*17*). Depending on the outcome of the test, the isolate can be assigned to one of several described serotypes. This classification is constantly being revised and updated based on the latest insights form biochemical and molecular genetic analyses of serotypes, and has currently reached a consensus of over 30 distinct serotypes (*18–26*), the most common in Europe being serotypes 7, 9 and 2 (*3*).

The structure and content of the bacterial capsule is genetically encoded in the *capsular polysaccharide biosynthesis* (*cps*) locus, a cluster of genes coding for a number of proteins required for transcriptional regulation and several steps in the biosynthesis of the capsule, including a capsular polysaccharide polymerase (*27*). Most differences between serotypes at this *cps* locus can be detected by polymerase chain reaction (PCR) (*28, 29*). A commonly accepted nomenclature defines a serotype by the corresponding *cps* locus detected via PCR. Accordingly, serotypes 2, 9 and 7 will be referred to as *cps2*, *cps9* and *cps7* throughout this manuscript.

Since the capsules of some serotypes seem to share surface epitopes and other serotypes share *cps* genes, neither serology (*19, 30*) nor genotyping by PCR (*31*) alone can accurately differentiate between some of the currently described serotypes. Multilocus sequence typing (MLST) allows for a more detailed and at the same time very robust analysis of *S. suis* isolates, because it allows for the comparison of differences between isolates at conserved genetic loci, like housekeeping genes. A now widely-used MLST model on the basis of seven *S. suis* housekeeping genes was introduced in 2002 by King *et al.* (*32*).

In a more recent approach, identification of serotypes is further complemented by analysis of the presence or absence of prominent virulence factors (e.g. SLY, MRP, EF) as an addition to PCR or MLST (*33–35*).

1.2. **Pathogenesis of *S. suis* infection in pigs**

Similar to other porcine pathogens, such as *Staphylococcus aureus (36), Haemophilus parasuis (37), Streptococcus dysgalactiae* and several species of *Lactobacillus (38)*, *S. suis* usually colonizes the mucosa of the upper respiratory tract, the urogenital tract and secondary lymphatic organs like lymph nodes and tonsils asymptomatically and is therefore considered a commensal (*39–44*). Already shortly after birth bacteria can be found in the saliva (*45*) and in secondary lymphatic organs like palatine and nasopharyngeal tonsils or mandibular lymph nodes of piglets (*39, 42, 46, 47*). Newborn piglets are usually infected because of high bacterial load in the vaginal tract (*43*), nasal or vaginal secretions, feces or milk (*47*) of the mother. While piglets carry different serotypes and strains of *S. suis* on their mucosa, only infections from a few virulent strains will cause disease (*43, 48*). Serotypes 1-9 are most prevalent in bacterial isolates from diseased pigs (*49–52*) and most serotypes are commonly found in the brain, joints and the lung (*53*). Transmission of these strains in breeding facilities may happen through vertical or horizontal transmission by direct contact or aerosol (*43, 46, 54*), by environmental contamination, like feed troughs (*55*), fomites like veterinary or farm equipment (*55, 56*), or even vectors like flies (*57*). Spread among piglets seems to be associated with excessive changes in environmental temperature, high relative air humidity, crowding, and groups of piglets with a difference in age of more than 2 weeks (*58*). A different tendency among serotypes to form aerosols has been reported as well (*59*).

When invasion of the blood by bacteria (i.e. bacteremia) is not controlled early by the immune system, it usually results in systemic inflammation which produces symptoms like fever, fatigue, loss of appetite and, in the case of a septic or toxic shock, low blood pressure. On a cellular level, the transition from bacteremia to septicemia is accompanied by a rapid first response of innate immune cells and subsequent cytokine release, which can trigger further cytokine release, recruitment of other innate and adaptive immune cells, and ultimately responses by T and B lymphocytes. Uncontrolled release of pro-inflammatory cytokines, a so-called cytokine storm, can result in multiple organ failure and death (*60*). The causes for cytokine storms are complex and not well understood.

1.2.1. **The immunological gap**

Suckling piglets are passively protected by immune cells as well as IgG antibodies received with the colostrum from the mother sow (*61*). Pigs start producing own antibodies only after weaning when maternal antibody levels have decreased, which results in a state of temporary deficiency in protective antibodies, also called the „immunological gap" (**Figure 1**). During this period piglets are especially prone to outbreaks of various diseases, such as meningitis, endocarditis, arthritis or septicemia, which can result in losses of up to 14 % of a pig herd (*39, 62, 63*). The development

of a serotype-independent *S. suis* vaccine to be applied early in life of piglets would help young pigs to mount a strong protective immune response against virulent *S. suis* right before or after weaning and prevent invasive infection.
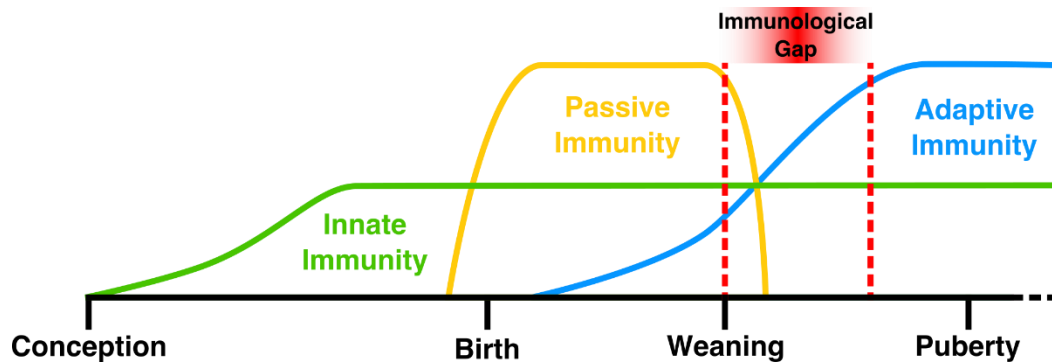

Figure 1 – Outline of the immunological gap in weaning piglets. The innate immune system develops in the embryonic stages. After birth, the piglet is passively protected by maternal antibodies which are taken up with the colostrum. During this period the piglet's own adaptive immunity starts to develop. After weaning at around 6 weeks of age, it takes another few weeks until the adaptive immune system is fully developed, which leaves the animal with an insufficient humoral protection. This period is called the „immunological gap". Modified after Butler *et al.*, 2007 (*64*), Axis not proportional.

During the weaning period, piglets undergo changes in the colonizing bacterial flora of the mucosa, especially of the tonsils and nasopharynx (*38, 42*). The porcine tonsils are considered to be one of the main portals of entry for *S. suis* resulting in systemic spread through blood or the lymphatic system (*46, 65, 66*). Accordingly, epidemic strains can survive in the tonsils for prolonged periods of time (*67*).

The mechanisms behind the transition from colonization to invasion are still poorly understood. Since dissemination of *S. suis* in pigs occasionally results in septicemia and death (*68, 65*), it is crucial to better understand the mechanisms and processes underlying colonization and invasion. However, the cellular, molecular or environmental factors required for the transition of colonization to invasion is still largely unclear.

1.2.2. **From colonization to invasion**

It is currently assumed that *S. suis* can cross the mucosa of the respiratory tract to become invasive (*68*). Tissue damage by induction of pro-inflammatory mediators at the epithelial barrier could be one mechanism for spread of invasive *S. suis* strains from mucosal surfaces. Dang *et al.* found that *S. suis* upregulates pro-inflammatory mediators like TNF, IL-6 and IL-8 on a transcriptional level when swine tracheal epithelial cells are co-infected with swine influenza virus (*69*). In a more mechanistical approach, Wang *et al.* then showed that swine tracheal epithelial cells are more prone to bacterial adhesion and invasion when pre-infected with swine influenza virus and also

induces TNF, IL-6 and IL-8 (*70*). This supports the theory that inflammation and tissue damage (each might be induced by co-infection) may promote the spread of *S. suis* systemically.

The most popular theory to explain the spread of *S. suis* to peripheral organs has been the "trojan horse theory", which states that bacteria are internalized, but not killed by monocytes, and can thereby pass the blood-brain barrier to cause meningitis (*71*). However, it is currently assumed that bacteria travel through the bloodstream extracellularly, avoiding phagocytosis through their bacterial capsule and possibly attaching to blood monocytes externally ("modified trojan horse theory") (*68, 72, 73*).

1.2.3. **Immune responses and immune evasion in invasive *S. suis* infection**

Since the innate immune system is the first line of defense against *S. suis*, it is crucial to understand the interactions of its bacterial components with immune cells. Virulence factors are diverse and it is still unclear which virulence factors exactly play an essential role in the pathogenesis of *S. suis*-associated disease, especially since virulence of *S. suis* seems to depend on the individual set of expressed virulence factors, which can vary greatly even between strains of the same serotype (*74*). However, especially structural components of the bacterial cell wall and capsule have been shown to be effectively sensed by cells of the innate and adaptive immune system.

The most effective mechanisms of killing of *S. suis* rely on an interplay of innate and adaptive immune responses, namely by antibody-mediated opsonization, (opsono-)phagocytosis by neutrophils and activation of the complement system (*75*). This is best illustrated by the immune evasion mechanisms of *S. suis*: It has been shown that *S. suis* can induce the formation of neutrophil extracellular traps (NETs) and evade trapping (*76*). Complement killing and opsonization can be impaired through expression of an immunoglobulin M-degrading enzyme which can degrade specific IgM (*77, 78*). The virulence factor suilysin is toxic to neutrophils and reduces opsonization and thereby complement-dependent killing of *S. suis*. These immune evasive properties as well as a decreased activation of pro-inflammatory mediators may be mediated by sialic acid (*79, 80*). The capsular polysaccharide (cps) is a heavily investigated virulence factor of *S. suis* with anti-phagocytic properties, which has been shown to play important roles in protection against killing by neutrophils and dendritic cells (DC) (*27, 81–86*), but is poorly immunogenic since it only induces low amounts of cps-specific antibodies (*87*). A study in human monocyte-derived dendritic cells showed that *S. suis cps2* was able to resist intracellular killing by DCs and modulate cytokine production towards a more anti-inflammatory profile compared to other serotypes (*88*). Furthermore, the bacterial capsule of a serotype 2 strain has been shown to play a partial role in impairing MHC-II-restricted antigen presentation of dendritic cells, and *S. suis* reduced production of IL-12p40, IL-2 and TNF-α, which are cytokines associated with a T helper

cell 1 (Th1) response (*89*). This is in line with another study in mouse splenocytes, which also showed that the cps limited the release of Th1-derived cytokines like TNF-α or IFN-γ (*90*).

1.2.4. **Cytokines and septicemia**

It has long been known that septicemia and more severe outcomes like sepsis in humans are generally associated with monocytes and macrophages producing exuberant levels of pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β, or IL-8 (*91–93*). In LPS-mediated septic shock, activation of monocytes and macrophages even leads to an increase of anti-inflammatory IL-10, which, however, is not able to control the increase in pro-inflammatory cytokines (*94*).

So far, studies on cytokine induction or sepsis after *S. suis* infection have mostly been done with whole blood and cell culture models from humans and mice. Studies investigating porcine blood are scarce and have mostly been done *in vitro* or in culture of peripheral blood mononuclear cells (PBMC).

Human macrophages and murine DCs can detect *S. suis* through TLR-2 and CD14 (*95, 96*), but *S. suis* can also trigger cytokine release by TLR-2- and MyD88-independent pathways (*97*). It was shown that cell wall components of *S. suis* stimulate porcine dendritic cells and induce IL-1β, IL-6, TNF-α, IL-12p40, and IL-8 (*98*). Also, the encapsulated wildtype strain was resistant to phagocytosis, but increases the expression of TLR-2 and -6. Taken together, this supports the notion of a two-sided character of the bacterial capsule, which is able to protect the bacteria against phagocytosis, but at the same time is effectively sensed by pattern recognition receptors (PRR) of the innate immune system.

Graveline *et al.* showed that *S. suis* capsule and cell wall can induce TNF-α, IL-6 and IL-1β in mouse macrophages in TLR-2-dependent and -independent ways (*95*). Segura and colleagues showed that *S. suis* can potently induce pro-inflammatory cytokines like IL-6, TNF-α, or IL-1β, first in murine macrophages (*99*), then in a human monocyte cell line (*100*), and finally in the first *in vitro* model for porcine whole blood (*101*). Later it was demonstrated for porcine DCs *in vitro* that *S. suis* not only impairs phagocytosis through its cps, but also induces classical pro-inflammatory cytokines, like IL-1β, IL-6, IL-8, IL-12p40 and TNF-α, mostly through cell wall components, and even reduces the expression of the costimulatory molecules CD80/86 and the antigen-presenting MHC-II complex, both of which are crucial for the activation of T cell immune responses (*98*). A co-infection study with PRRSV showed that IL-6 and TNF-α are synergistically upregulated in bone marrow-derived DCs (BMDC) when additionally infected with *S. suis* (*102*).

Prior to our study *in vivo* data on the relationship between bacteremia, the induced cytokines and their cellular sources in pigs were still missing.

1.3. **Aim of the study**

The aim of this work was to analyze the cytokine response to *S. suis* infection in the blood compartment to understand how bacteremia in piglets is linked with the release of pro- and anti-inflammatory cytokines. Another aim was to compare the *in vivo* situation of bacteremia with *in vitro* models, and to investigate the potential effects of cytokines on bacterial killing. Specifically, the serum levels of TNF-α, IL-6, IFN-, IL-17A, and IL-10 in pigs intravenously infected with *S. suis* were analyzed. Based on *in vivo* data, the cytokine production in whole blood and PBMC was analyzed, and cellular sources of the induced cytokines were evaluated. Furthermore, by concurrent analysis of *S. suis* survival, as well as TNF-α neutralization or addition of recombinant TNF-α, the potential effects of cytokine release on bacterial killing in blood were investigated.

2. MATERIAL AND METHODS

2.1. **Material and Animals**

2.1.1. **Bacterial strains**

The *cps2* strain 10 (*103*) and the isogenic capsule mutant strain 10cpsΔEF (*27*) were kindly provided by Hilde Smith (Wageningen, GE, The Netherlands). The *cps7* strain 13-00283-02 and the *cps9* strain 16085/3b have been characterized previously in whole-blood killing assays and confirmed to be virulent in experimental infections with piglets (*104, 105*).

2.1.2. **Blood and serum donors**

Weaning piglets as blood donors were 7 to 12-week-old German Landrace pigs conventionally bred and held at Lehr- und Versuchsgut Oberholz, Großpösna, Germany or purchased from BHZP GmbH, Dahlenburg-Ellringen, Germany. Piglets in the *in vivo* infection experiment were bred at Lehr- und Forschungsgut Ruthe, Ruthe/Sarstedt, Germany.

2.1.3. **Chemicals**

Unless stated otherwise, common laboratory chemicals were purchased from ROTH (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany), or Applichem (Darmstadt, Deutschland).

Table 1 - List of used commercial chemicals with manufacturers.

Chemical	Manufacturer
Diff Quik®	Medion Diagnostics AG, Düdingen, FR, Switzerland
Gentamicin	Merck KGaA, Darmstadt, Germany
Gelatine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
KPL TMB Microwell Peroxidase	KPL, Gaithersburg, USA
Penicillin	Merck KGaA, Darmstadt, Germany
Streptomycin	Merck KGaA, Darmstadt, Germany
Trypan Blue	Sigma Aldrich, Munich, Germany
Türk's solution	Merck KGaA, Darmstadt, Germany

2.1.4. **Consumables**

Table 2 – List of used consumables with manufacturers.

Consumable	Manufacturer
96 well micro plates, flat-bottom	Techno Plastic Products, Trasadingen, Switzerland
96 well micro plates, U-bottom	Techno Plastic Products, Trasadingen, Switzerland
96 well micro plates, V-bottom	Laborfachhandel Dr. Ilona Schubert, Leipzig, Germany
Biocoll (1.077 g/ml)	Merck KGaA, Darmstadt, Germany
Conical centrifuge tubes (50 ml, 15 ml)	Fisher Scientific GmbH, Schwerte, Germany
Cryo tubes with external thread (1.2 ml)	Techno Plastic Products, Trasadingen, Switzerland
Dennison Avery adhesive covers	Laborfachhandel Dr. Ilona Schubert, Leipzig, Germany
Disposable pipette tips (10 µl, 250 µl)	Rainin, Gießen, Germany
Disposable pipette tips (200 µl, 1,000 µl)	Laborfachhandel Dr. Ilona Schubert, Leipzig, Germany
Disposable syringes with Luer-Lok™ tips (1 ml, 50 ml)	BD Biosciences, Heidelberg, Germany
Falcon™ polystyrene round-bottom tubes (5 ml)	Fisher Scientific GmbH, Schwerte, Germany
Fetal bovine serum Good Forte (batch P161107)	PAN-Biotech GmbH, Aidenbach, BY, Germany
Iscove's Modified Dulbecco's Medium	PAN-Biotech GmbH, Aidenbach, BY, Germany
MaxiSorp™ round-bottom micro plates	Fisher Scientific GmbH, Schwerte, Germany
Monovette® Luer for serum (#02.263, 9 ml)	Sarstedt AG&Co, Nümbrecht, Germany
Monovette® Luer with Lithium heparin (#02.265, 9 ml)	Sarstedt AG&Co, Nümbrecht, Germany
MS columns	Miltenyi Biotech, Bergisch Gladbach, Germany
Oxoid™ Columbia agar plates with 5% sheep blood	Fisher Scientific GmbH, Schwerte, Germany
Polysine® slides	Fisher Scientific GmbH, Schwerte, Germany
Reaction tubes (2 ml, 1.5 ml, 0.5 ml)	Eppendorf, Hamburg, Germany

Shandon™ EZ single Cytofunnel™ for Cytospin™ centrifuge	Fisher Scientific GmbH, Schwerte, Germany
Sterican® disposable cannulas (25 G x 1''/30G x ½'')	Braun-Melsungen, Melsungen, Germany
Sterile inoculation loops (plastic)	Laborfachhandel Dr. Ilona Schubert, Leipzig, Germany
Todd-Hewitt broth	BD Biosciences, Heidelberg, Germany
Tryptic soy broth without Dextrose	BD Biosciences, Heidelberg, Germany

2.1.5. **Devices and software**

Table 3 – List of used devices with manufacturers.

Model	Function	Manufacturer
Analytical devices		
BioPhotometer 6131	Spectrophotometer	eppendorf AG, Hamburg, Germany
LSRFortessa™	Flow cytometer	BD Bioscience, Heidelberg, Germany
Spectra-Max 340PC384	Microplate reader	Molecular Devices, Sunnyvale, USA
Synergy™ H1	Microplate reader	BioTek Instruments, Inc., Winooski, USA
Centrifuges and rotators		
5417R, centrifuge	Centrifuge, refrigerated	eppendorf AG, Hamburg, Germany
GMC-060	Centrifuge	LMS Co., Ltd., Tokyo, Japan
Heraeus Megafuge 40R	Centrifuge, refrigerated	Fisher Scientific GmbH, Schwerte, Germany
Heraeus Primo R	Centrifuge, refrigerated	Fisher Scientific GmbH, Schwerte, Germany
Shandon™ Cytospin™ 4 Cytocentrifuge	Centrifuge, refrigerated	Fisher Scientific GmbH, Schwerte, Germany
Stuart SB3	Rotator	Cole-Parmer, Staffordshire, United Kingdom
Stuart SRT6	Roller mixer	Cole-Parmer, Staffordshire, United Kingdom
Cooling and heating units		

BE600	Incubator	Memmert GmbH + Co. KG, Schwabach, Germany
Heraeus Function Line B20	Incubator	Fisher Scientific GmbH, Schwerte, Germany
Premium NoFrost	Freezer	Liebherr, Bulle, Germany
Thermo® Cryotechnics STINOL KG103	Fridge/freezer combination	Fisher Scientific GmbH, Schwerte, Germany
Thermo® Forma® Series II Water Jacketed CO2 Incubator	Incubator	Fisher Scientific GmbH, Schwerte, Germany
Thermo® Forma™ -86°C ULT Freezer	Deep freezer	Fisher Scientific GmbH, Schwerte, Germany
Titramax 1000/Inkubator 1000	Incubator, shaker	Heidolph Instruments GmbH & CO. KG, Schwabach, Germany
UF 755 GG	Deep freezer	B Medical Systems, Hosingen, Luxemburg
Pipets		
Bel-Art™ Vaccu-Pette	Autoclavable 96-well pipetting device	Fisher Scientific GmbH, Schwerte, Germany
eLINE® (50 - 1,000 µl)	Electronic pipet	Merck KGaA, Darmstadt, Germany
Multipette® plus	Multi-step pipet	eppendorf AG, Hamburg, Germany
Pipet-Lite™ (100 - 1,000 µl)	Mechanical pipets	Rainin, Gießen, Germany
Pipet-lite™ (20-300 µl)	Multi-channel pipet (12-channels)	Rainin, Gießen, Germany
pipetus®	Electronic pipet for serological tips	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Research® (0.5 - 10 µl, 10 - 200 µl, 100 - 1,000 µl)	Mechanical pipets	eppendorf AG, Hamburg, Germany
Miscellaneous		
Carl Zeiss™ Axiovert™	Microscrope, inverted	Fisher Scientific GmbH, Schwerte, Germany
Herasafe, Class 2, Type H laminar flow bench	Laminar flow bench	Fisher Scientific GmbH, Schwerte, Germany
Mini Vortexer	Vortexer	VWR®, Darmstadt, Germany
Neubauer-improved counting chamber	Counting chamber	Paul Marienfeld GmbH & Co.KG, Lauda Königshofen, Germany

VarioMACS separator	Magnetic cell sorting device	Miltenyi Biotech, Bergisch Gladbach, Germany

Table 4 – List of used software with developers.

Software	Purpose	Developer
BD FACSDiva™, flow cytometry recording software	Flow cytometry recording software	BD Bioscience, Heidelberg, Germany
FlowJo, v.10.6.0, flow cytometry analysis software	Flow cytometry analysis software	FlowJo LLC, Ashland, USA
GraphPad PRISM v5.01	Data analysis software	GraphPad Software, Inc., San Diego, USA
SoftMax Pro, v5.0	Microplate reader software	Molecular Devices, LLC, San Jose, USA

2.2. **Methods**

2.2.1. **Growth and preparation of bacterial strains**

- <u>PBS (pH 7.4)</u>: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 12.9 mM Na_2HPO_4

Bacteria suspensions for stimulation were grown from frozen bacterial stocks stored at – 80 °C in 15% glycerol (v/v in PBS). Frozen bacteria were transferred from the cryo tube to a Columbia agar plate containing 5% sheep blood (Fisher Scientific) using a sterile plastic inoculation loop.

Single colonies were obtained using the fractional smear method: With a clean inoculation loop, the bacterial suspension was spread out in a single, waving line over one fifth of the plate. Then another clean inoculation loop was used to spread part of the first smear over another fifth of the plate, and so on. This gradual thinning is needed to spread the bacterial suspension enough for single colonies to grow on the sheep blood agar. The plated bacteria were incubated over night at 37 °C and 5% CO_2.

On the next day, a single colony was picked with a sterile disposable plastic inoculation loop and resuspended in 5 ml of sterile THB in a 15 ml centrifuge tube. This liquid culture was incubated for 9 hours at 37 °C and 5% CO_2 with a loose screw cap to allow gas exchange between the bacterial culture and the incubator.

Bacterial growth in a liquid culture can be measured indirectly using the optical density (OD) of the culture medium. While light can pass through a clear liquid like water unhindered, a medium containing proliferating bacterial cells will increase in opacity over time and therefore absorb

light increasingly. The optical density can be measured using a spectrophotometer and is expressed as a dimensionless value, with a value close to 0 for clear water and increasing values with higher absorbance of light.

After 9 hours of incubation, the optical density of the liquid bacterial culture was measured at 600 nm (OD_{600}). The bacterial suspension was then diluted to an OD_{600} of 0.03 in 60 ml of pre-warmed THB in a glass flask and incubated at 37 °C and 5% CO_2 until an OD_{600} of 0.6 was reached, which is equivalent to the late exponential growth phase of *S. suis* containing an optimal concentration of viable bacteria.

The resulting bacterial suspension at OD_{600}=0.6 was then pelleted by centrifugation at 3,200 x g for 15 min at 4 °C. The supernatant was discarded. Aliquots of bacteria used in whole blood (bactericidal assay) were stored in THB with 15% sterile glycerol (v/v in PBS). Bacteria used for stimulation of PBMC culture were washed twice with sterile PBS (centrifugation at 3,200 x g, 15 min, 4 °C). The washed pellet was resuspended in PBS with 15% sterile glycerol, distributed into 500 µl aliquots and stored at -80 °C.

Since cells were stimulated with thawed aliquots of bacterial suspensions, the bacterial concentration of the aliquots was not determined with fresh bacterial suspensions, but after one freeze-thaw cycle. A frozen aliquot was thawed on ice and diluted up to 1:10^6 in sterile PBS in a 1:10 dilution series with 6 steps. The dilution was carried out in 200 µl of volume in sterile 96-well micro plates (U-bottom, Techno Plastic Products, Trasadingen, Switzerland). From each dilution 20 µl were plated in duplicates on Columbia agar plates with 5% sheep blood. The droplet was run across the plate by gravity ("running droplet" method). After overnight incubation at 37 °C and 5% CO_2 the average bacterial colony count from each dilution was calculated. The colony count was then calculated back to the bacterial concentration of the stock using a weighted arithmetic mean according to the formula described by Bast (*106*): $m = \frac{10^{-x}}{v} * \frac{\sum c_x + \sum c_{x+1}}{n_x + 0.1\, n_{x+1}}$. m = weighted arithmetic mean; n = number of plates; 10^{-x} = dilution factor of the lowest dilution; v = volume used for plating (here 20 µl); $\sum c_x$ = total number of colonies on all plates (n_x) of the lowest dilution 10^{-x}; $\sum c_{x+1}$ = total number of colonies on all plates (n_{x+1}) of the next higher dilution $10^{-(x+1)}$.

2.2.2. Animal experiments and blood sampling

All piglets used in this study were from conventional herds with carriers of numerous *S. suis* genotypes. Whole blood samples for *in vitro* experiments were drawn from *Vena cava cranialis* from healthy piglets between 6 and 10 weeks of age (German Landrace, weaned at four weeks of age) in 9 ml lithium-heparin monovettes (Sarstedt AG&Co, Nümbrecht, Germany). Porcine blood was taken in accordance with the permit no. N19/14, approved by the responsible authorities of the state of Saxony, Germany (Landesdirektion Sachsen, Chemnitz, Germany).

In vivo data were generated from samples of an independent infection experiment carried out at the University of Veterinary Medicine Hannover, Germany (permit No. 33.8-42502-04-18/2879, Committee on Animal Experiments of the Lower Saxonian State Office for Consumer Protection and Food Safety) with German Landrace piglets from a different herd. The handling and treatment of animals was in strict accordance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, as well as the German Animal Protection Law.

Eight-week-old German Landrace piglets (weaned at four to five weeks of age) were intravenously infected under ketamine and azaperone anesthesia with 3×10^8 cfu of *S. suis cps2* strain 10 (*103*). The goal of the infection was to induce meningitis, but no arthritis or shock symptoms. The infection dose was based on experience with previous intranasal and intravenous infection studies in piglets of the same age group with *S. suis* serotype 2 and serotype 9, respectively. PBS was intravenously injected in the control animals under same conditions. Twelve hours post infection, the piglets were anaesthetized through application of ketamine and azaperone, and anesthesia was maintained for six hours (i.e., from 13 to 19 h after intravenous infection) via inhalation of isoflurane while blood was drawn. Blood samples were taken from *Vena jugularis* or *Vena cava cranialis* pre-infection and from *Arteria femoralis*, respectively, at defined time points (0, 13, 16 and 19 h post infection) and filled into serum and plasma (lithium-heparin) monovettes (Sarstedt AG&Co, Nümbrecht, Germany, NW, Germany).

From Li-heparin blood, serial dilutions were plated on Columbia blood agar and the bacterial load (cfu/ml) was determined after incubation at 37 °C for 20 h. In addition, serum samples were taken, centrifuged 30 min after blood withdrawal, and immediately frozen after centrifugation in small volumes in liquid nitrogen and stored at -80 °C until usage. Colostrum-deprived serum was obtained from piglets that were born dead without showing signs of decay or from very weak, newborn piglets that were euthanized immediately after birth due to the inability to rise and to take up colostrum.

2.2.3. Isolation of PBMC and granulocytes by density gradient centrifugation

- Erythrocyte lysis buffer: 0.155 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM di-sodium EDTA

PBMC were isolated from whole blood by density gradient centrifugation with Biocoll (1.077 g/ml; Merck KGaA, Darmstadt, Germany). Granulocytes were collected from the pellet of a density gradient separation of whole blood with Biocoll (described above), and again subjected to a second Biocoll density gradient separation. Erythrocytes were removed by incubation in erythrocyte lysis buffer for 5 min on ice.

Control of separation purity of granulocytes by histological staining

To determine the purity of granulocytes, the separated fraction was transferred to glass slides (2 x 10^5 cells/slide) with a Cytospin™ 4 centrifuge at 1,000 rpm centrifugation for 3 min (Fisher Scientific GmbH) and stained with a Diff Quik® staining kit (Medion Diagnostics AG, Düdingen, Switzerland) according to the manufacturer's instructions with 5 dips in each Diff Quik Fix solution, Diff Quik I solution, and Diff Quik II solution. The frequencies of lymphocytes, monocytes and granulocytes (neutrophils, eosinophils and basophils) out of 100 total cells were determined microscopically with a Carl Zeiss™ Axiovert™ (Fisher Scientific GmbH). The purity of granulocytes determined with this method was 96% (median, **Figure 6C**). The viability of granulocytes was confirmed by cell count in 0.4% trypan blue solution.

2.2.4. **Separation of monocytes by magnetic cell sorting (MACS)**

- <u>PBS (pH 7.4)</u>: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 12.9 mM Na_2HPO_4
- <u>MACS buffer</u>: 0.5% BSA (v/v), 2 mM EDTA in PBS

Magnetic cell sorting relies on magnetic beads which are coupled to antibodies specific to typical surface markers of cell populations. Monocytes of humans and pigs express the marker CD14 on their surface (*107–111*) which can be detected with commercially available CD14-specific beads. When the CD14-expressing cells are labelled with the magnetic beads, the cell suspension can be rinsed through a column consisting of magnetized particles. The unlabelled cells are washed out of the column while all cells labelled with the magnetic CD14-beads are retained by the magnetic particles (positive selection). In the next step, the column is demagnetized and the labeled cells with the magnetic CD14-beads can be washed out of the column.

For magnetic cell separation of monocytes, PBMC were labeled with cross-reactive anti-human CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions and separated on MACS® MS columns (Miltenyi Biotec GmbH) in the magnetic field of a VarioMACS separator.

Since antibodies can cross-react with non-specific epitopes, the purification of monocytes had to be validated. The main priority of the experiment was to separate CD14-expressing monocytes and CD3-expressing T cells, which can be distinguished by flow cytometry through fluorescent labelling of characteristic surface markers.

Control of separation purity of the CD14-positive fraction by flow cytometry

To check the purity of the monocyte fraction after separation, the number of monocytes and T cells was determined by flow cytometry in the unseparated PBMCs and in the CD14-positive monocyte fraction. Cells were stained with a viability dye (eFluor™ 506, Fisher Scientific GmbH, Schwerte, Germany), a T cell-specific anti-CD3 antibody (clone BB23-8E6-8C8; Becton Dickinson

GmbH, Heidelberg, Germany), and an antibody against the myeloid marker CD172a ("SWC3", clone 74-22-15A; Becton Dickinson GmbH). Since commercially available antibodies against CD14 for use in flow cytometry did not perform well, this method was chosen to distinguish between T cells and myeloid cells in flow cytometry.

2.2.5. **Cell counting**

To determine cell numbers for cell culture, cell suspensions were diluted with Trypan blue (Sigma Aldrich, Munich, Germany) and given onto a Neubauer counting chamber. Trypan blue accumulates in damaged or dead cells, but is excluded from viable cells. The number of viable cells in a suspension can therefore be determined by counting unstained cells. Viable cells were counted in 4 diagonal small squares across each of the four large corner squares to determine the average number of cells in one large square (16 small squares). Since the depth of the chamber between glass slide and cover slip is 0.1 mm and the area of one large square is 1 mm^2, the volume above one large square is equal to 0.1 mm^3 or 0.1 μl. Therefore, multiplying the cell count of one large square with the factor 10^4 and with the dilution factor of the cell suspension gives the cell number per ml.

To determine the cell number in whole blood, Türk's solution (Merck KGaA, Darmstadt, Germany) was used instead of Trypan blue to remove erythrocytes during counting. Türk's solution contains acetic acid, which results in hemolysis of residual erythrocytes. The gentian violet dye in Türk's solution stains all leukocytes and allows no evaluation of cell viability.

2.2.6. **Cell culture and stimulations**

PBMC culture

Isolated PBMC were seeded into sterile 96-well flat-bottom plates at 10^6 cells/well in 200 μl Iscove's Modified Dulbecco's Medium (IMDM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum(FBS, PAN-Biotech GmbH), 50 μg/ml gentamicin (Merck KGaA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Pen/Strep; Merck KGaA). Viable *S. suis* strains from glycerol stocks were added at PBMC-to-bacteria ratios of 10:1 or 1:1 (corresponding to 10^5 and 10^6 cfu/well), as indicated in the respective figure descriptions and legends. After 42 h of stimulation with *S. suis*, supernatants were taken and stored at -20 °C.

Separation experiments

For separation experiments, PBMC were cultivated at 10^6 cells/well (200 μL). CD14-positive monocytes were cultivated at 5 x 10^4 cells/well (5% of PBMC); granulocytes were cultivated at 1 x 10^6 cells/well. For stimulation, the same final concentration of 10^6 cfu/well was used for all three fractions (equivalent to a 1:1 ratio of PBMC to bacteria).

IL-10 neutralization experiments

For IL-10 neutralization, anti-porcine IL-10 (clone 148801; R&D Systems Inc.) or isotype control mIgG2b (clone 20116; R&D Systems Inc.) were added at 0.1 µg/ml. Bioactivity of clone 148801 has been demonstrated previously (*112*).

2.2.7. **Flow cytometry**

- <u>PBS (pH 7.4)</u>: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 12.9 mM Na_2HPO_4
- <u>Flow cytometry buffer</u>: 3% FBS (PAN-Biotech GmbH), 0.1% sodium azide (v/v) in PBS
- <u>Paraformaldehyde (PFA)</u>: 2% (v/v) in flow cytometry buffer

Staining

Cells were transferred to 96-well v-bottom microplates (Laborfachhandel Dr. Ilona Schubert, Leipzig, Germany) and washed twice with PBS (380 x *g*, 3 min, 4 °C). Unless stated otherwise, cells were stained at 4 °C in darkness. After washing, cells were centrifuged at 380 x *g* for 3 min at 4 °C, the supernatant was discarded and the pellet was resuspended by shaking for 30 s on a microplate shaker/incubator.

To assess cell vitality, cells were stained in 50 µl of an amine-reactive live/dead dye (eFluor™ 506, Fisher Scientific GmbH, Schwerte, Germany) in PBS for 25 min. Amine-reactive dyes penetrate dead or damaged cells and bind to free amines in the cytoplasm. The dye is excluded by live cells and therefore allows a discrimination between viable and dead cells. For staining of surface markers, the cells were washed twice with flow cytometry buffer. Cells were then stained in 40 µl of the corresponding antibody (CD3*PE-Cy7, clone BB23-8E6-8C8; CD172a*FITC, clone 74-22-15A; both from BD Biosciences) for 15 min. For each antibody, a corresponding isotype staining was included to control for unspecific staining. For fixation, cells were washed twice in PBS and then incubated in 2% PFA for 15 min. Cells were finally washed and then resuspended in flow cytometry buffer until flow cytometric measurement.

Flow cytometric analyses

Flow cytometric measurements were carried out on an LSR Fortessa™ flow cytometer (BD Biosciences). Flow cytometric data were analyzed with FlowJo software, version 10.6.0 (TreeStar, Ashland, USA).

2.2.8. **Bactericidal assay**

Bactericidal assays were conducted as previously described (*78*) with some modifications. Briefly, 500 µl of heparinized blood was mixed with *S. suis* strains at a concentration of 2 x 10^6 cfu/ml (time point t_0) and incubated at 37 °C for 2 or 6 h on a rotator at eight rounds per minute (rpm). To study the time course of TNF-α induction and bacterial survival, whole blood from each piglet was mixed at room temperature with *S. suis* for a final concentration of 2 x 10^6 cfu/ml. Infected

blood was immediately distributed to separate reaction tubes for each time point (0, 30, 60, 90, and 120 min; 500 µl each) and incubation at 37 ℃ on a rotator (8 rpm) was started simultaneously for all tubes.

To calculate survival factors, blood was diluted and incubated on Columbia agar in duplicate (running droplet). Colony-forming units (cfu) were counted after overnight incubation at 37 °C. The survival factor at a given time point t_x was calculated as quotient of cfu at t_x divided by cfu at t_0. For functional studies, recombinant porcine TNF-α (10 ng/ml) or neutralizing anti-TNF-α antibody (4 µg/ml; both R&D Systems Inc., Minneapolis, MN, USA) was added. Bioactivity of recombinant TNF-α has been demonstrated previously (*113*).

For a serum-reconstituted bactericidal assay, whole blood was washed twice with PBS and afterwards supplied with half the initial blood volume of defined sera (e.g., from colostrum-derived pigs).

2.2.9. **Quantification of cytokines by ELISA**

- <u>PBS (pH 7.4)</u>: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 12.9 mM Na_2HPO_4
- <u>Washing buffer</u>: 0.05% Tween®20 (v/v) in PBS
- <u>Blocking buffer</u>: 0.1% gelatine (v/v), 0.5% BSA (w/v) in PBS
- <u>Serum diluent</u>: 0.1% gelatine (v/v), 0.5% BSA (w/v), 0.05% Tween®20 (v/v) in PBS
- <u>H_3PO_4 (1M)</u>

ELISA kits for porcine IL-6, IL-10, and TNF-α were purchased from R&D Systems Inc (Minneapolis, USA). Ninety-six-well plates (Nunc MaxiSorp™ round-bottom; Fisher Scientific) were coated with antibodies according to the manufacturer's instructions. The biotinylated detection antibodies were coupled with a streptavidin–horseradish peroxidase conjugate (R&D Systems, Inc.) for 20 min at room temperature. The peroxidase was developed with a 3,3',5,5'-Tetramethylbenzidin (TMB) solution (SeraCare, Milford, MA, USA, formerly KPL) and the reaction was stopped after 20 min with H_3PO_4. Optical density (OD) values were measured with a SpectraMax 340PC384 microplate reader (Molecular Devices, LLC, San Jose, CA, USA) at 450, and at 630 nm as a background reference, and analyzed with SoftMax® Pro v5.0 software (Molecular Devices, LLC).

Establishment of an ELISA for the quantification of porcine IL-17A

Since no porcine IL-17A ELISA kits were available commercially, this ELISA was established with a monoclonal anti-porcine IL-17A antibody as coating antibody (clone MT504, Mabtech AB, Nacka Strand, Sweden) and a polyclonal rabbit anti-porcine IL-17A serum for detection (catalog no. KPB0499S, Kingfisher Biotech, Inc., Saint Paul, USA), and a porcine IL-17A standard. Incubation times and reagents were adopted from protocols of commercially available cytokines ELISA kits. A washing step consisted of 3 washes with 250 µl washing buffer each.

Protocol:

> Ninety-six-well plates (Nunc MaxiSorp™ round-bottom; Fisher Scientific) were coated with 50 μl of the coating antibody diluted in PBS and incubated over night at RT. On the next day, the plate was washed an incubated with blocking buffer for 1 hour at room temperature to block unspecific binding sites on the plate. After a washing step, the standard was diluted in serum diluent and incubated in 50 μl for 2 hours at RT. The blocking buffer was washed and then 50 μl of the detection antibody diluted in serum diluent was added and incubated for 2 hours at RT. The detection antibody was washed and the detection antibody was coupled to a streptavidin-horseradish peroxidase conjugate (50 μl/well, 1:200 in serum diluent) and incubated for 20 min at RT. After a final washing step, 100 μl of the peroxidase substrate, TMB, was added. After approximately 15 min the reaction was stopped with 50 μl of 1 M H_3PO_4. OD values were detected at 450 (signal) and 630 nm (background).

First, the performance of the coating antibody was evaluated at 5, 2.5, and 1.25 μg/ml with the detection body used at 2.0, 1.0, and 0.5 μg/ml. Porcine IL-17A (MoBiTec GmbH, Göttingen, Germany) was used as a standard in a 1:2 dilution series from 2,000 to 31.25 ng/ml. The standard was detected linearly down to 125 pg/ml with a coating antibody concentration of 5 μg/ml independently of the concentration of the detection antibody (**Figure 2**). Lower coating antibody concentrations resulted in non-linear OD_{450} measurements.

Finally, the optimal concentration of the detection antibody and the detection limit were determined. The detection antibody was used in a 1:2 dilution series from 0.5 to 0.062 μg/ml; the standard was used in a 1:2 dilution series from 1,000 to 0.6766 ng/ml. A sigmoidal curve was measured at a concentration of 0.5 μg/ml of the detection antibody (**Figure 2**).
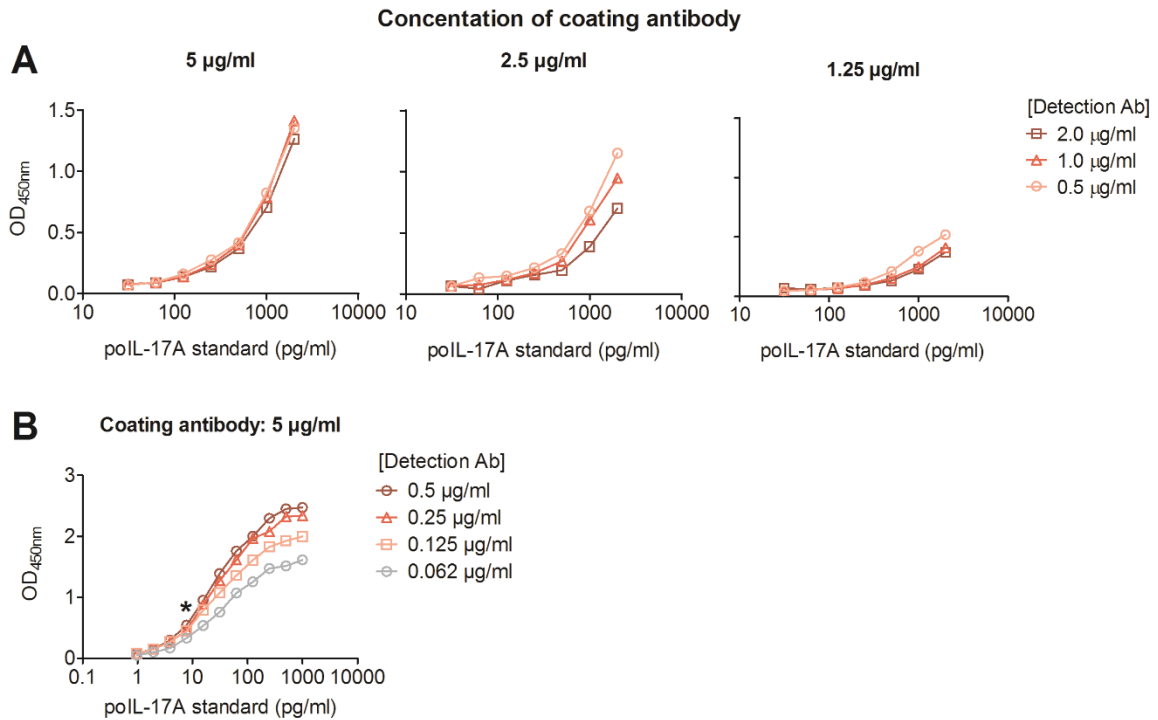
Figure 2 – Establishment of an ELISA for porcine IL-17A. The performance range of the monoclonal coating antibody (clone MT504, Mabtech AB, Nacka Strand, Sweden) and the polyclonal rabbit anti-pig IL-17A antibody (Kingfisher Biotech, Inc., Saint Paul, USA) using a porcine IL-17A standard (MoBiTec GmbH, Göttingen, Germany). (A) First, the optimal concentration of the coating antibody was tested (5, 2.5, 1.25 µg/ml) using three different concentrations of the detection antibody (2.0, 1.0, 0.5 µg/ml). At a concentration of 5 µg/ml the coating antibody detected the standard at a sufficiently high OD. Concentrations of poIL-17A standard: 2,000, 1,000, 500, 250, 125, 62.5, 31.25 pg/ml. (B) Using the coating antibody at the determined concentration of 5 µg/ml, the detection antibody was tested at lower concentrations (0.5, 0.25, 0.125, 0.062 µg/ml). At 0.5 µg/ml the IL-17A standard was detected optimally. Concentrations of poIL-17A standard: 1,000, 500, 250, 125, 62.5, 31.125, 15.625, 7.8125, 3.9063, 1.9531, 0.9766 pg/ml. The detection limit of the established poIL-17A ELISA is 7.8125 pg/ml (*).

2.2.10. Quantification of *S. suis*-specific Immunoglobulin (Ig) G by ELISA

- 5% skim milk (v/v): skim milk powder in ddH$_2$O
- Citrate buffer: 0.003% H$_2$O$_2$ (v/v), 500 µg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

Round-bottom 96-well plates (Nunc MaxiSorp round-bottom; Fisher Scientific) were coated with *S. suis* strain 10 and blocked with 5% skim milk solution. After incubation with control and sample sera, *S. suis*-specific IgG was detected with a polyclonal goat anti-pig IgG antibody (catalog no. A100-105P, Bethyl Laboratories, Inc., Montgomery, USA) coupled to peroxidase. The peroxidase was developed with a citrate buffer containing 0.003% H$_2$O$_2$ and 500 µg/ml of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate. OD values were measured with a Synergy™ H1 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 and at 630

nm as background reference. IgG was quantified in relative units (RU) as a ratio of the samples relative to a control serum, as described previously (*66*).

2.2.11. **Statistics**

Data analyses were performed with GraphPad Prism v5.01 (GraphPad Software, Inc., La Jolla, CA, USA) to calculate normal distribution. Statistical significance (α = 0.05) was calculated with a parametric test for normally distributed data (Student's t-test) and with non-parametric tests for data that was not normally distributed (Mann-Whitney test, Kruskal-Wallis test and Dunn's post-hoc test).

3. RESULTS

3.1. **Detection of IL-6 and IL-10 in sera of piglets with pronounced bacteremia after intravenous infection with *S. suis***

The induction of inflammatory cytokine responses upon streptococcal infection has been demonstrated in previous studies in human whole blood and also by bacterial stimulation in murine *in vitro* models (*99, 114, 115*). Previously, *S. suis* has been shown to induce pro-inflammatory cytokines in a study with porcine whole blood (*101*). This study investigated whether *S. suis* induces cytokines during bacteremia *in vivo* after an intravenous infection that mimics bacteremia caused by an invasive infection. Cytokine levels were determined in serum samples taken from nine piglets (weaned at four to five weeks of age; six to ten weeks old at time of analysis) at 0, 13, 16, and 19 h (time points given by the animal permit), six of which were intravenously infected with *S. suis* strain 10 and three of which served as uninfected controls. To prevent stress-induced immunomodulation during blood sampling, all experimental animals were under anesthesia from 13 to 19 h post infection when blood was drawn. Three of six infected individuals developed pronounced bacteremia 13 h post infection (hpi; **Figure 3A**). Two of three pigs with pronounced bacteremia showed an increase in serum IL-6 (**Figure 3B**): individual H7 showed an IL-6 peak of 1.9 ng/ml at 16 hpi, and individual H2 a peak of 0.9 ng/ml at 19 hpi. For IL-10, an increase of serum levels with pronounced bacteremia was also detectable in two of three piglets: piglet H2 showed an increase up to 1.6 ng/ml at 19 hpi. Piglet H3, which did not show elevated IL-6 levels, showed an IL-10 peak of 0.7 ng/ml at 16 hpi. The piglet H7 did not show increased IL-10 secretion. In contrast, no increase in IL-6 or IL-10 levels was detectable in the sera of three pigs, which were able to control bacteremia, as well as in the three uninfected control pigs. In addition, TNF-α, IL-17A and IFN-γ were measured in the serum of all experimental animals, but were not detectable during this time frame.

In summary, it was found that during infection, cytokines are induced *in vivo* in piglets with pronounced *S. suis* serotype 2 bacteremia. However, it remained open whether TNF-α was induced at earlier time points after infection.
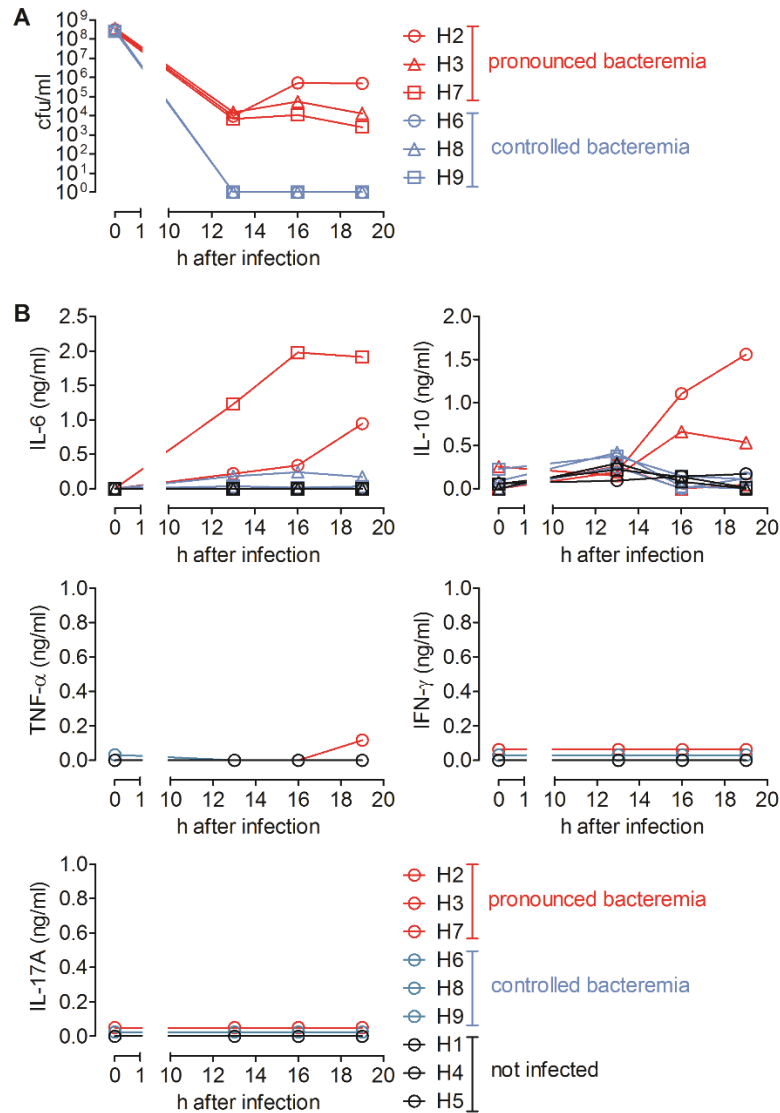

Figure 3 - Detection of IL-6 and IL-10 post intravenous *S. suis* infection in sera of piglets with pronounced bacteremia. (A) Quantification of bacterial load in blood 0, 13, 16, and 19 h after intravenous infection of six piglets (eight weeks old, weaned at four weeks of age).z (B) Cytokine levels in individual sera measured by ELISA before and after infection with *S. suis* strain 10 (*cps2*, n = 6) and in serum of non-infected control animals (n = 3). Note that earlier time points than 13, hpi could not be taken due to the experimental setup. Some of the bacterial load data shown here were additionally used in another study, though in context with other parameters (*116*).

3.2. *In vitro* induction of pro- and anti-inflammatory cytokines by *S. suis* is limited by IL-10

As an induction of IL-6 and IL-10 was observed in piglets with pronounced bacteremia, the next step was to test whether cytokine induction could be reproduced in a whole-blood assay upon addition of viable *S. suis*, as has been demonstrated previously (*101*). Since IL-6 and TNF-α are commonly induced immediately after infection (*99, 101*), cytokines were measured in plasma at earlier time points (2 and 6 h) after stimulation with *S. suis* than determined in the *in vivo*-infection experiment. To assess the proliferation or killing of *S. suis* in the whole-blood assay, bacterial survival was determined after 2 and 6 h. Whole blood of eight to nine-week-old piglets was stimulated with the same bacterial strain from the infection experiment (strain 10, *cps2*). As a further control, an acapsular mutant of the encapsulated WT strain 10 was used, described as 10cpsΔEF (*27*), that is known to show an enhanced cytokine release by *S. suis* (*101, 95*), since the capsule covers the immune-stimulatory cell wall components. Strain 10 induced TNF-α and IL-6 after 2 h and at significantly increased levels after 6 h, while IL-10 was only induced 6 h after stimulation (**Figure 4A**). The magnitude and time course of the cytokine induction for the acapsular strain was very similar. In contrast to similar cytokine response, the acapsular strain was killed after 2 h, whereas the wild type (WT) strain 10 proliferated within 2 and 6 h after infection (**Figure 4B**).

In summary, the *in vivo* data could be confirmed in a whole-blood assay (**Figure 3**) by showing that *S. suis* strain 10 induces pro-inflammatory (i.e., IL-6) and anti-inflammatory (i.e., IL-10) cytokines in blood. In addition, an early TNF-α induction was detected (2 h). Previous findings, that acapasular *S. suis* induces similar cytokine levels, but shows a reduced survival in a porcine whole blood (*101*), could also be confirmed.
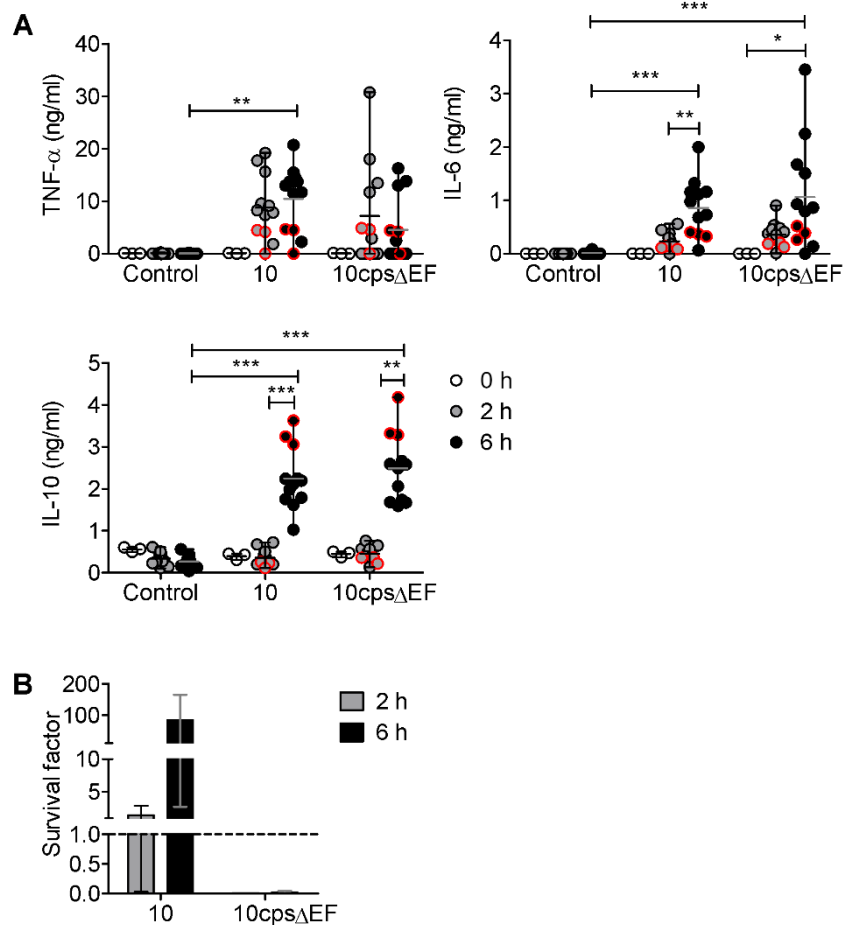
Figure 4 - The induction of pro- and anti-inflammatory cytokines by encapsulated and acapsular *S. suis* strain 10 *in vitro* in whole blood is similar, but bacterial survival is different. (A) Quantification of cytokines in plasma before (0 h, n = 3) and 2 or 6 h after (n = 12) the addition of 2 x 10^6 colony-forming units (cfu)/ml *S. suis* strain 10 or 10cpsΔEF to whole blood. Median (horizontal line) of all samples are shown; red symbols indicate the individuals that are shown in B. (B) Bacterial survival from the same samples 2 and 6 h after *in vitro* infection. Calculation of survival factors (tx) = (cfu at t_x)/(cfu at t_0). Bars show median and range of individually calculated survival factors (n = 3). Graphs show data of two independent experiments with eight to nine-week-old piglets. Statistical analysis was conducted by Kruskal–Wallis test and Dunn's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Bacterial proliferation and/or neutrophil-mediated killing of *S. suis* in the used whole-blood assay can change the ratio of bacteria to blood cells and complicate the interpretation of data. Thus, to exclude bacterial proliferation and to maintain a defined ratio of bacteria to blood cells in the following experiments, isolated PBMC were used in a cell culture system with antibiotics to analyze *S. suis*-induced cytokine production. To prevent phagocytosis and killing as much as possible PBMC were used, which no longer contain granulocytes, since granulocytes are important for bacterial killing (*75*). PBMC, isolated from healthy weaning piglets, were stimulated with the WT *S. suis* strain 10 and the acapsular mutant. In PBMC the induction of TNF-α, IL-6 and IL-10 by the WT strain was dependent on the bacterial load, but resulted in only marginal cytokine

levels (**Figure 5A**). In contrast to WT *S. suis*, cytokine induction after stimulation with the acapsular strain was significantly higher, but IL-6 and IL-10 also showed a dose-dependent increase, whereas TNF-α was already strongly induced at a PBMC-to-bacteria ratio of 10:1.
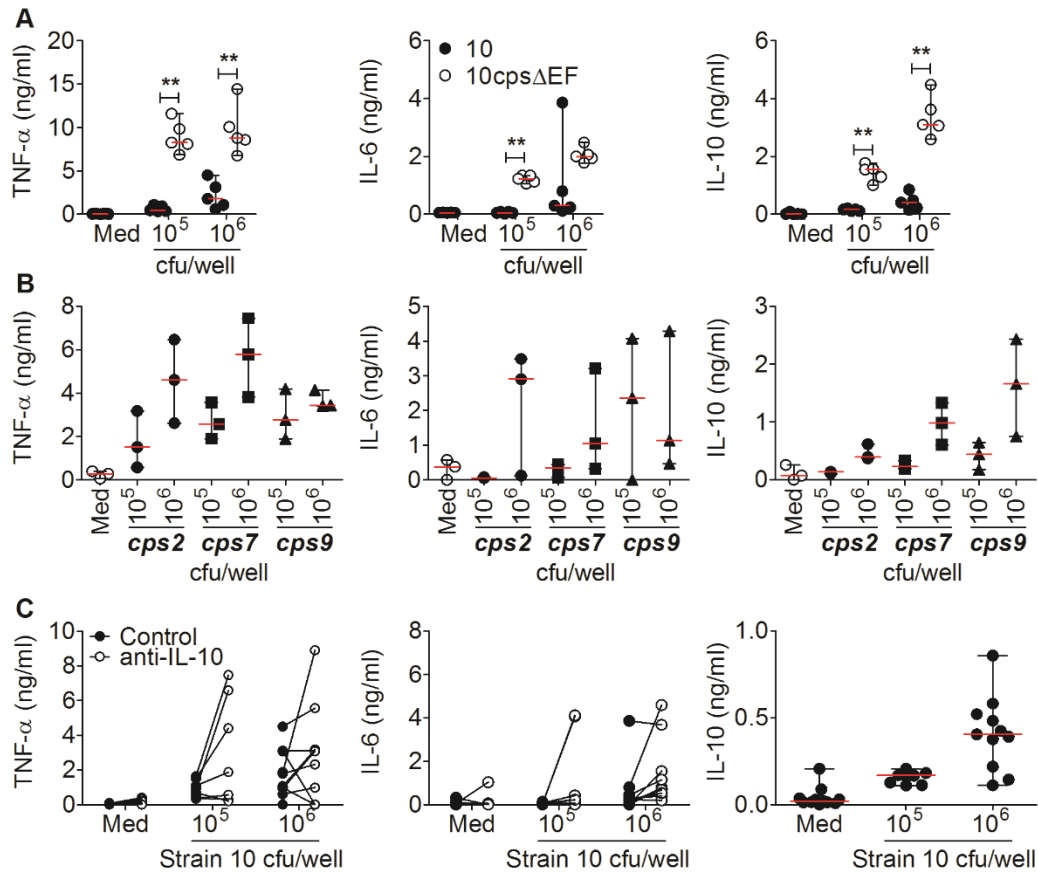

Figure 5 - Cytokine release by peripheral blood mononuclear cells (PBMC) is *S. suis* dose-dependent, and IL-6 and TNF-α secretion is limited by IL-10. (A) Isolated porcine PBMC were stimulated with strain 10 or the acapsular mutant strain 10cpsΔEF at a PBMC-to-bacteria ratio of 10:1 (10^5 cfu/well) or 1:1 (1×10^6 cfu/well) in the presence of antibiotics. Cytokines were measured in cell culture supernatants after 42 h of incubation (n = 5). Median and individual pigs shown. (B) Cytokine levels in supernatants of PBMC stimulated with *S. suis* strains 10 (*cps2*), 13-00283-02 (*cps7*) or 16085/3b (*cps9*) at the PBMC-to-bacteria ratios 10:1 and 1:1 in the presence of antibiotics. Cytokines were measured after 42 h of incubation (n = 3). (C) TNF-α and IL-6 production in presence of neutralizing anti-IL-10 antibody or with an isotype control after the stimulation of porcine PBMC with *S. suis* strain 10 at the same PBMC-to-bacteria ratios in the presence of antibiotics. IL-10 production was determined for reference. Cytokines from supernatants were measured after 42 h of incubation (n = 9–11). Graphs show pooled data from four independent experiments with seven to ten-week-old piglets. For statistical analyses, Mann Whitney test was performed (** p < 0.01).

In summary, cytokines were induced by stimulation with the WT strain, dependent on the high bacterial load and on the dose-dependent induction of IL-6 and IL-10 by the acapsular strain. The observed dependency on the bacterial load is in line with the previous findings (**Figure 4**), since a high bacterial load of the WT strain 6 h upon infection (**Figure 4B**) was associated with higher cytokine induction (**Figure 4A**). Moreover, the association found *in vivo* for cytokine production

and pronounced bacteremia (**Figure 3**) underlines the bacterial dose-dependency observed *in vitro* using either whole blood or PBMC.

To test if the results obtained using the *cps2* strain 10 are representative of different *S. suis* serotypes, two further *S. suis* strains with clinical relevance, but different serotypes were included: the *cps7* strain 13-00283-02 (*104*) and the *cps9* strain 16085/3b (*105*). TNF-α was induced at similar levels by all three strains and increased dose-dependently up to 6 ng/ml (**Figure 5B**). IL-6 induction was highly donor-dependent and varied between 0 and 4 ng/ml in all three strains. The IL-10 induction was also dependent on the bacterial load between 0.1 and 1.8 ng/ml by all three strains. This shows that *S. suis* strains of different serotypes induce TNF-α, IL-6 and IL-10 similarly.

IL-10 is a potent anti-inflammatory mediator. To investigate a possible regulatory effect of *S. suis*-induced IL-10 on the production of the pro-inflammatory cytokines IL-6 or TNF-α, both cytokines were measured following *S. suis* stimulation of PBMC in the presence of a neutralizing anti-porcine IL-10 antibody. Compared to the control, IL-10 neutralization increased the levels of TNF-α and IL-6 (**Figure 5C**). Although the differences were not significant, a trend emerged comparing the PBMC of individual piglets in the absence and presence of neutralizing anti-IL-10 antibodies.

These findings suggest that *S. suis*-induced IL-10 might reduce the production of the pro-inflammatory cytokines IL-6 and TNF-α.

3.3. **Encapsulated *S. suis* induces TNF-α in monocytes, but not in granulocytes**

Cells of the innate immune system, such as monocytes, macrophages and neutrophils, have been shown to be producers of TNF-α, IL-6 and IL-10 upon stimulation with bacterial antigens in mice and humans (*100, 114, 117, 118*). To determine the contribution of myeloid cells of the blood to the production of the detected cytokines, porcine PBMC, CD14-positive monocytes isolated from the PBMC and granulocytes from the blood of pigs from the same herd were stimulated with WT *S. suis* strain 10 or with the acapsular mutant, and measured cytokines in the supernatants.

The purity of the CD14-positive fraction was assessed by flow cytometry. Since anti-CD14 antibodies did not perform sufficiently and did not allow to distinguish between CD14-negative and CD14-positive populations, the separation purity was instead assessed with the myeloid marker CD172a as a frequency of all CD3-negative cells (**Figure 6A**). The frequency of myeloid cells was enriched to >95% in the CD14-positive fraction, compared to ~5% of myeloid cells in the unseparated PBMC fraction (**Figure 6B**).

Granulocytes were isolated from the pellet after two density gradient centrifugations of whole blood. The purity of the granulocyte pellet was assessed microscopically after staining for eosinophilic and basophilic structures using a Diff Quik® staining kit by Medion Diagnostics AG, Düdingen, Switzerland. The granulocyte pellet contained >95% neutrophils (**Figure 6C**).
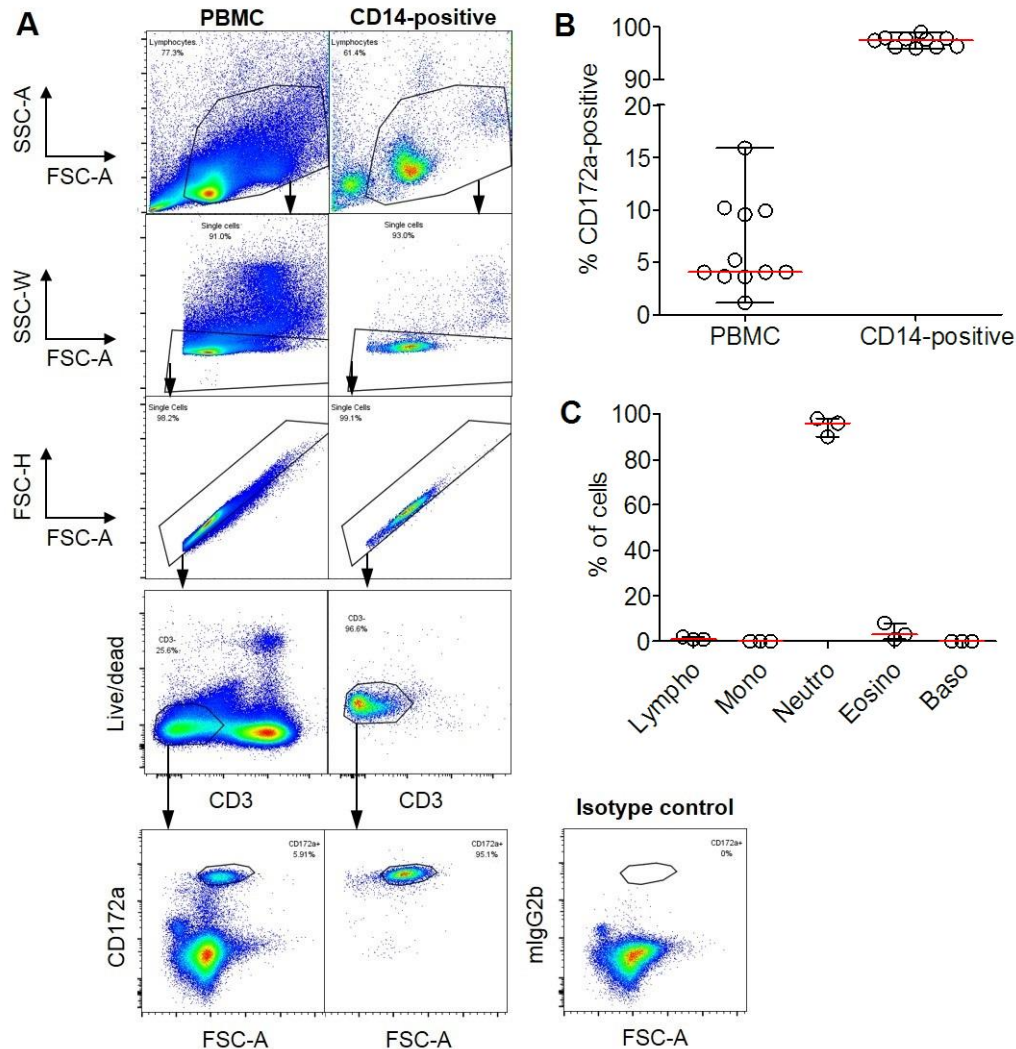

Figure 6 – Validation of separation purity of monocytes by flow cytometry and of granulocytes by microscopy. (A) Flow cytometry gating for the quantification of CD172a-positive cells. (B) Plot of frequency of CD172a-positive cells as percentage of viable CD3-negative cells in unseparated PBMC and in the CD14-positive fraction after magnetic cell separation for all 11 donors. (C) Separation purity of granulocytes after density gradient centrifugation for all 3 donors. Representative cells from the granulocyte pellet were transferred to a glass slide and stained with the Diff Quik® staining kit (Medion Diagnostics AG, Düdingen, Switzerland). Cells were counted microscopically.

The blood leukocytes of pigs contain up to 50% granulocytes and 5 – 10% monocytes (*119*). *In vitro* cell numbers of PBMC, CD14-positive monocytes and granulocytes were chosen accordingly: PBMC – 1×10^6 cells/well (200 µl), monocytes – 5×10^4 cells/well, granulocytes – 1×10^6 cells/well. For adequate comparison of PBMC, isolated monocytes and granulocytes, the same *S. suis*

concentration of 1 x 10^6 colony-forming units (cfu)/well was used to stimulate all cell fractions (equivalent to a 1:1 ratio of unseparated PBMC to bacteria).

The PBMC fraction, containing lymphoid cells and monocytes, produced low amounts of TNF-α (median 0.3 ng/ml), IL-6 (median 0.1 ng/ml) and IL-10 (median 0.2 ng/ml) in response to WT *S. suis*, and high amounts (median of TNF-α: 14.5 ng/ml, IL-6: 1.0 ng/ml, IL-10: 1.7 ng/ml) in response to the acapsular mutant (**Figure 7A**). The monocyte fraction produced clearly detectable levels of TNF-α (median 0.7 ng/ml) after stimulation with the WT strain and high levels of TNF-α (median 4.0 ng/ml) with the acapsular strain. IL-6 and IL-10 were detectable in monocytes stimulated with the acapsular strain (median IL-6: 0.4 ng/ml, IL-10: 0.6 ng/ml), but not after stimulation with the WT strain. Neither IL-6 nor IL-10 were detectable in granulocytes when stimulated with the WT strain (**Figure 7B**). Stimulation with the acapsular strain induced TNF-α (max. 3.3 ng/ml, median 0.42 ng/ml) in granulocytes, but at lower levels than in stimulated monocytes, whereas IL-6 and IL-10 induction in granulocytes was detectable only at a very low level in some samples (**Figure 7B**).

In summary, it was demonstrated that monocytes are the main myeloid producers of TNF-α induced by encapsulated WT *S. suis*.
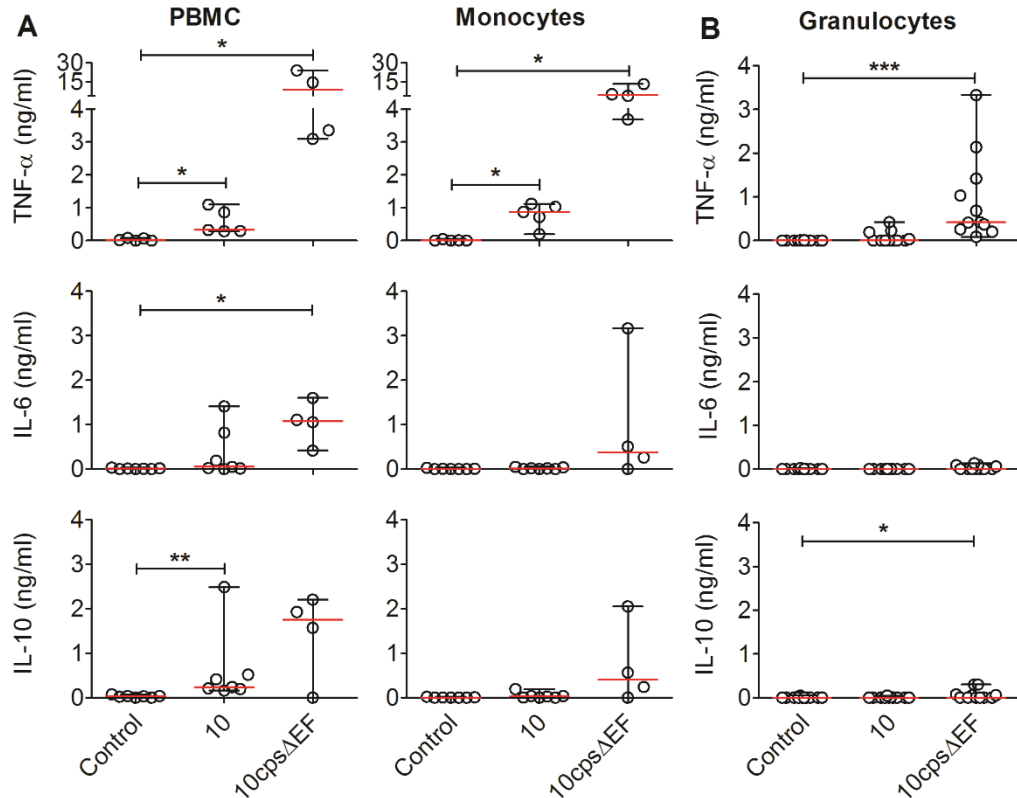

Figure 7 - Monocytes are the main producers of TNF-α in response to encapsulated *S. suis*. (A) Levels of TNF-α, IL-6 and IL-10 secreted by PBMC and CD14-positive monocytes after stimulation with the WT *S. suis* strain 10 (n = 4–

7) or the acapsular mutant strain 10cpsΔEF for 42 h in the presence of antibiotics. PBMC were cultivated at 1 x 10⁶ cells/well (200 μL) and monocytes were cultivated at 5 x 10⁴ cells/well. Bacteria were always used at 1 x 10⁶ cfu/well, which is equivalent to a PBMC-to-bacteria ratio of 1:1. Graphs show pooled data of four independent experiments with seven to 10-week-old piglets. Depending on the resulting cell yield of the monocyte separation, fractions were stimulated with *S. suis* strain 10 and/or 10cpsΔEF. (B) Density gradient-purified granulocytes (1 x 10⁶ cells/well) from different donors, but pigs of the same herd (n = 11) were stimulated under the same conditions as the PBMC and the monocytes. The cytokines were quantified from cell culture supernatant by ELISA. Graphs show data from two independent experiments with seven to 10-week-old piglets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ according to Mann Whitney test of control (medium) versus *S. suis*-stimulated samples.

3.4. **TNF-α induction is not crucial for bacterial killing of *S. suis in vitro*, not even in the absence of *S. suis*-specific immunoglobulin (Ig) G**

It was demonstrated that *S. suis* induces pro- and anti-inflammatory cytokines *in vivo* (**Figure 3**), in whole blood (**Figure 4**) as well as in PBMC and in isolated monocytes (**Figure 5, Figure 7**). The most prominent cytokine detected in the *in vitro* assays is TNF-α, since it is produced in high amounts in the whole-blood assay even by the encapsulated WT strain 10 (**Figure 4**). Whether *S. suis*-induced TNF-α is able to affect bacterial killing remains elusive.

TNF-α secretion was induced rapidly after bacterial contact (2 h, **Figure 4A**). To determine the time point of bacterial killing and TNF-α release more precisely, a kinetic analysis in whole blood was performed in 30 min intervals after *in vitro* infection with WT *S. suis* strain 10 (2 × 10⁶ cfu/ml). TNF-α levels in plasma and bacterial survival factors were determined at these time points. TNF-α was induced only after 90 min of stimulation, but bacteria were already killed after 30 min (**Figure 8A**).
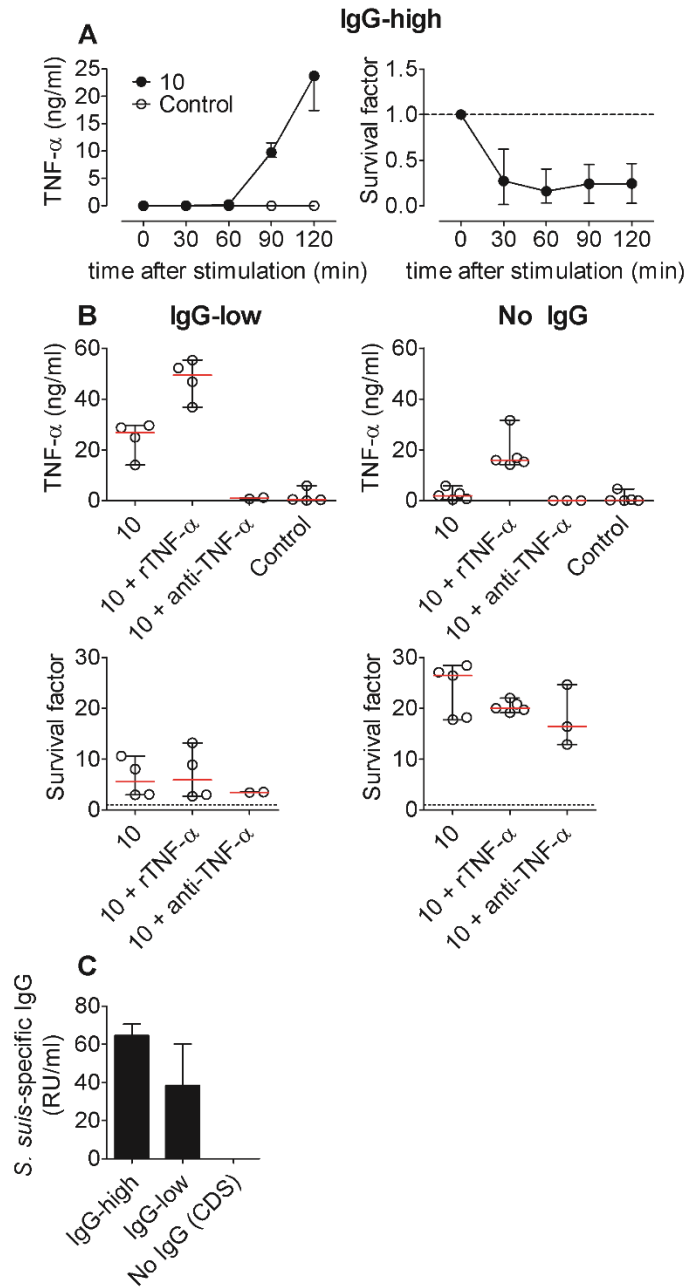
Figure 8 - TNF-α does not contribute to bacterial killing *in vitro*, not even in the absence of *S. suis*-specific antibodies. (A) Kinetics of TNF-α induction and bacterial survival after infection of whole blood with 2×10^6 cfu/ml *S. suis* strain 10 (n = 3). (B) Blood samples of piglets with low specific IgG (n = 2–4) or blood cells reconstituted with colostrum-deprived serum (CDS, see Section 4.2 Methods) (no specific IgG; n = 3–5) were infected with the viable *S. suis* strain 10 alone or additionally treated with porcine recombinant TNF-α (rTNF-α, 10 ng/ml) or neutralizing anti-TNF-α antibody (4 µg/ml). Bacterial survival was determined after 2 h. (C) Relative units per mL (RU/ml) of *S. suis* strain 10-specific IgG in sera of different groups of piglets. RU were calculated relative to a reference serum. Blood and sera were categorized based on the amount of *S. suis* strain 10-specific IgG: IgG-high > 60/ml RU (n = 3); IgG-low < 60 RU/ml (n = 4); No IgG = 0 RU/ml, using CDS with no detectable *S. suis* strain 10-specific IgG antibodies for the reconstitution of porcine blood cells. Graphs show data of four independent experiments with six to eight-week-old piglets.

Detection of *S. suis* (strain 10)-specific IgG from plasma samples of the whole blood revealed a high antibody background (>60 relative units/ml, defined as "IgG-high", **Figure 8C**) in these blood samples. This suggests that TNF-α does not contribute to bacterial killing in this experimental setup, but that *S. suis*-specific antibodies are critical for the rapid killing. Therefore, whole blood samples with low *S. suis*-specific IgG (<60 RU/ml, defined as "IgG-low", **Figure 8C**) were chosen and, in a further approach, blood cells were reconstituted with colostrum-deprived serum (CDS, named "no IgG") to completely exclude the interfering effects of antibody-mediated mechanisms on bacterial killing. To investigate the effects of TNF-α in more detail, recombinant TNF-α (rTNF-α, 10 ng/ml) or a neutralizing anti-TNF-α antibody were added to the *S. suis*-stimulated blood assay. In whole blood of the IgG-low group, the addition of rTNF-α did not increase bacterial killing (**Figure 8B**, left panel). To investigate the effect of endogenous TNF-α on killing, a neutralizing anti-TNF-α antibody was additionally used. The neutralization of endogenous TNF-α also did not change bacterial survival. In the approach with reconstituted porcine blood cells with CDS, *S. suis* induced TNF-α only marginally (**Figure 8B**, right panel). As expected, *S. suis* showed better survival in the CDS background compared to the IgG-low background. However, bacterial killing was not affected by the addition of rTNF-α.

In conlusion, under the chosen experimental conditions, TNF-α does not contribute to the killing of *S. suis* in blood, even not in the absence of specific antibodies.

4. DISCUSSION

S. suis is an important pathogen in the piglet farming industry. The pathogenesis of *S. suis* is linked to invasive infection resulting in septicemia, meningitis, arthritis, or endocarditis (*46*). Bacteremia as a potential mechanism for dissemination of this pathogen goes along with the manifold clinical symptoms. Whether bacteremia provokes the release of cytokines in the natural host of *S. suis* was the focus of this study by an experimentally induced invasive infection.

An induction of IL-6 and the anti-inflammatory mediator IL-10 could be detected after 13 – 19 hpi in serum of intravenously *S. suis*-infected piglets that showed a pronounced bacteremia. By *in vitro* stimulation with *S. suis*, the *in vivo* results were confirmed showing that *S. suis* induces TNF-α and IL-6 following *in vitro* stimulation of porcine blood. Similar to the *in vivo* data, increased cytokine production accompanied the growth of *S. suis* in the whole-blood assay and in PBMC stimulated with higher doses of bacteria. Thus, the data demonstrate that increased bacterial load with *S. suis* in the blood leads to increased cytokine production. This shows the relevance of the whole-blood assay as an *in vitro* system and confirms earlier findings (*101*) using a porcine whole-blood assay to mimic some aspects of bacteremia. Furthermore, the presented study demonstrates the survival and growth of *S. suis* in blood despite the presence of pro-inflammatory cytokines IL-6

and TNF-α. Therefore, these pro-inflammatory cytokines do not appear to be crucial for killing mechanisms. Specifically, TNF-α did not play a role in bacterial killing in whole blood *in vitro* when recombinant TNF-α or a neutralizing antibody against TNF-α were added, not even in conditions with low *S. suis*-specific antibodies. In addition, it was shown that the induction of IL-10 limits the production of the pro-inflammatory cytokines TNF-α and IL-6.

4.1. **Effects of cytokine induction by *S. suis* in blood**

Cytokine kinetics

The induced cytokines detected in this study (**Figure 3, Figure 4**) match the pro-inflammatory cytokine profile described in the *in vitro* study in porcine blood by Segura *et al.* (*101*). In addition, the cytokine levels by stimulation with different *S. suis* serotypes are comparable (**Figure 5B**) (*101*). The differences found in the onset and magnitude of cytokine production *in vitro* may be related to genetic differences between the pig breeds and differences in procedures of *in vitro* experiments. The sequential onset of cytokine production, starting with TNF-α and followed by IL-6 and IL-10, has also been found in other studies (*101, 95, 120, 121*). A systemic infection of mice with an intraperitoneally administered suilysin-positive virulent serotype 2 strain led to a detectable TNF-α increase in serum between 3 and 6 hpi, but to a decline at 6 hpi, similar to TNF-α levels before infection (*115*). Due to experimental restrictions by the animal permit for the *in vivo* infection experiment (**Figure 3**), serum cytokine levels between 1 and 13 h post infection could not be analyzed in the presented study. Therefore, it is possible that a putative early peak of TNF-α (1 to 13 h) has been missed.

Impact of bacterial capsule on cytokine induction

In the whole-blood assay, the acapsular strain induced cytokines at a high magnitude although it was killed rapidly, while the WT strain induced comparable cytokine levels, but was not killed (**Figure 4**). The WT strain can evade killing through its bacterial capsule, which has been shown to effectively inhibit phagocytosis (*81–83, 85*). The higher proliferation of the WT strain results in an increased and prolonged exposure of immune cells to bacterial antigens. However, the capsule does not only mask surface-associated bacterial antigens, but exposed cell wall components are also more potent cytokine inducers than the capsule (*101, 95*). Previous studies in whole blood and human cell culture have demonstrated that cell wall components of *S. suis* can induce TNF-α and IL-6, depending on the CD14 receptor (*100*) and on toll-like receptor (TLR) 2 (*95*). Therefore, the acapsular strain induces cytokines at a higher magnitude (*101, 95*).

Effects of induced cytokines on bacteremia

The current understanding is that killing of *S. suis* is dependent on phagocytosis (*75*) and mediated by opsonizing antibodies. Segura *et al.* showed that opsonization of the *S. suis* serotype 2 with

specific immune serum reduced induction of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, associated with decreased bacterial survival ([101]). The observed *in vivo* induction of IL-6 and IL-10 in bacteremic pigs (**Figure 3**) raises the question of whether the induction of pro-inflammatory cytokines also has protective properties in bacteremia. However, it has been shown that decreasing IL-6 levels are associated with a protective effect in pneumococcal septicemia in pigs ([122]). Furthermore, a co-infection study in porcine bone marrow-derived dendritic cells (BMDC) experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV) and secondary infection with *S. suis* showed that phagocytosis of bacteria was impaired when IL-6 and TNF-α were upregulated ([102]), pointing to a detrimental effect of IL-6 or TNF-α upregulation. This is in line with earlier studies demonstrating a detrimental effect of pro-inflammatory cytokines during systemic inflammatory conditions, like bacteremia, sepsis or septic shock ([91, 92, 123, 124]). Therefore, the induction of IL-10 by *S. suis* and the observed limiting effect of IL-10 on TNF-α and IL-6 secretion (**Figure 5C**) can be interpreted as a physiological negative feedback to counteract an uncontrolled pro-inflammatory immune response and to ultimately allow for reversion to a state of immunological equilibrium. However, this feedback can have detrimental effects, too.

The infection of mice with influenza virus has been shown to trigger increased IL-10 levels in convalescent animals, but ultimately resulted in an increased susceptibility to *S. pneumoniae* infection and a lethality of 100% ([125]). Since it has been shown that porcine PBMC also highly upregulate IL-10 after PRRSV infection ([126]), a similar scenario could be possible in the field in pigs infected with PRRSV and a secondary invasive *S. suis* infection. The detrimental effects of high IL-10 induction have been further demonstrated for *Klebsiella pneumoniae* ([127]) and meningococcal infection ([128]), and could result in reduced phagocytic and bactericidal activity in neutrophils ([129]).

The presented data demonstrate that a potential negative impact of IL-10 on *S. suis* killing by downregulation of TNF-α is unlikely, since TNF-α was not involved in bacterial killing *in vitro*. Since TNF-α is also known as an inducer of oxidative burst in neutrophils, *in vivo* this cytokine could be able to support antibody-mediated killing mechanisms against streptococci, which are catalase-negative and therefore sensitive to reactive oxygen species ([130]). On the other hand, TNF-α has long been known to also have negative effects on the outcome of which has also been demonstrated by several studies investigating TNF-α neutralization as a treatment against septic shock and bacteremia ([131–133]). Therefore, TNF-α may contribute to an inflammatory response detrimental to the porcine host. However, in a pig model of *S. pneumoniae* sepsis, it was directly demonstrated that neutralization of TNF-α has no protective anti-inflammatory effect ([134]).

Individual differences in cytokine production

The magnitude of cytokine responses to *S. suis* infection varied notably between individuals, *in vivo* (**Figure 3**) and *in vitro* (**Figure 5, Figure 7**). In humans, single nucleotide polymorphisms (SNP), in the *TNF-α, CD14,* or *TLR* genes have been shown to have a considerable impact on the outcome of sepsis *(135, 136, 60)*. It is possible that pigs with high levels of cytokines have polymorphisms which make them especially prone to overshooting inflammation. Furthermore, inflammation and coagulation are associated and can amplify each other, so an individual's tendency for coagulation could be associated with the outcome of sepsis (*137–139*).

In summary, a high bacterial antigen load seems to be associated with the induction of IL-6 and TNF-α, and could cause an inflammatory cascade during bacteremia, potentially contributing to a detrimental outcome of invasive *S. suis* infection, especially since TNF-α did not contribute to bacterial killing in whole blood *in vitro*. For a more detailed understanding of the role of cytokines during an invasive infection in pigs, histopathological scoring could be of advantage to investigate the local detrimental effects induced by pro-inflammatory cytokines.

4.2. **Cellular sources of induced cytokines**

Monocytes separated from PBMC with magnetic anti-human CD14 beads were identified as the main producers of TNF-α by comparing cytokine levels released by PBMC and isolated monocytes after the addition of *S. suis* (**Figure 7**). Monocytes possess numerous pattern recognition receptors and could be more reactive to *S. suis* due to a previously described extracellular binding of *S. suis* to monocytes in the blood stream, which is independent of phagocytosis (*82, 72*). This bacterial attachment to phagocytic cells without internalization was also demonstrated for the murine macrophage cell line J774 (*73*) and release of pro-inflammatory cytokines like IL-6 and TNF-α by human monocytes in response to *S. suis* has previously been demonstrated using the human monocyte cell line THP-1 (*100*).

Monocytes in humans can be divided based on their expression of the LPS co-receptor, also known as CD14, and the Fcγ receptor III (FcγRIII), also known as CD16 (**Table 5**): CD14++ CD16- classical monocytes, CD14++ CD16+ intermediate monocytes, and CD14+ CD16++ non-classical monocytes (*140–142*) , the latter two subsets sometimes simply being referred to as CD16+ monocytes. It is suspected that that those three subsets represent transitional stages during an immune response (*143, 144*). In pigs, a CD14low CD163high population of monocytes has been deemed equivalent to the intermediate population in humans based on transcriptional profiling (*145*).

Name of monocytes[1]	Human[1]	Mouse[1]	Pig[2]	Cytokine expression[3]	Alternative name
Classical	CD14^{++} CD16^{-}	Ly6C^{++} CD43^{+}	CD14^{high} CD163^{low}	IL-6^{high}	"inflammatory" monocytes
Intermediate	CD14^{++} CD16^{+}	Ly6C^{++} CD43^{++}	CD14^{low} CD163^{high}	TNF-α^{high}	/
Nonclassical	CD14^{+} CD16^{++}	Ly6C^{+} CD43^{++}	not described	TNF-α^{high}	"patrolling"/ „resident" monocytes

Table 5 – Overview of blood monocyte nomenclature in humans, mice and pigs. Table modified after Ziegler-Heitbrock *et al.*, 2010 (*140*).

[1] according to the Nomenclature Committee of the International Union of Immunologic Societies (*140*).

[2] according to Fairbairn *et al.*, 2013 (*145*)

[3] according to Chimen *et al.*, 2017 (*146*)

Porcine monocytes respond to bacterial antigens in a similar way to human and murine monocytes by detecting PAMPs in a TLR-dependent manner and upregulating pro- and anti-inflammatory cytokines, including IL-1β, IL-6, TNF-α, IL-12p70, and IL-10 (*95, 118*). Therefore, the detected TNF-α production and the absence of IL-6 production (**Figure 7**) suggest that the isolated monocytes studied above are most similar to the non-classical/intermediate fraction in humans.

However, cytokine induction *in vivo* is probably a far more complex process requiring cell types that are missing in the *in vitro* model. For example, and there is evidence suggesting that endothelial cells (EC) can integrate and relay pro-inflammatory cytokine signaling, possibly by interaction with monocytes (*146, 147*). According to Chimen *et al.*, nonclassical/intermediate monocytes may secrete high levels of TNF-α in proximity of endothelial cells (EC), which in turn upregulate pro-inflammatory genes and also E-selectin (*146*) facilitating neutrophil recruitment. To counteract this pro-inflammatory response, classical monocytes then secrete IL-6, which in this context has anti-inflammatory effects by overriding the activating effects of TNF-α, reducing E-selectin expression in EC through SOCS3 and ultimately reducing neutrophil recruitment. Furthermore, a study on functional reprogramming of monocytes during sepsis showed that monocytes secrete high levels of cytokines like IL-6 and IL-10 during sepsis and transition to a more immunosuppressive state during recovery of the same patients, which points to the importance of "regulatory" IL-6 and IL-10 induction during sepsis and a controlled return to a non-inflammatory state for host survival. Sepsis and mortality in invasive *S. suis* infection in pigs may therefore result from an imbalance of the activities of those monocyte subsets, possibly through an overshooting pro-inflammatory cascade (bias towards nonclassical/intermediate

monocytes, TNF-α^{high}), while recovering pigs also have a controlled inflammatory TNF-α response from nonclassical monocytes, but manage to control the inflammation through induction of IL-10 and a multi-factorial "regulatory" effect IL-6.

IL-10 was not produced by monocytes or granulocytes but to varying amounts (0.25 – 2 µg/ml) by PBMC when stimulated with the encapsulated or the acapsular strain (**Figure 5**). Müller-Alouf *et al.* (*148*) demonstrated that human PBMC produce 0.5 to 1.5 ng/ml of IL-10 when stimulated with heat-killed group A streptococci for 48 hours. Furthermore, IL-10 production was increased substantially by stimulation with the superantigen exotoxin A, demonstrating that IL-10 is produced in a strongly lymphocyte-dependent manner. This is in line with the presented data showing moderate amounts of IL-10 only in the PBMC fraction.

Furthermore, in the presented study TNF-α production was strongly reduced by the reconstitution of blood with CDS as compared with sera containing high and low levels of *S. suis*-specific IgG (**Figure 8**), which suggests that opsonization by antibodies is important for TNF-α production, possibly for phagocytosis by granulocytes. Since phagocytosis of *S. suis* is impaired by the polysaccharide capsule (*82*) and granulocytes only secreted TNF-α *in vitro* when stimulated with the acapsular mutant (**Figure 7**), it is plausible that cytokine induction in granulocytes is dependent on phagocytosis. Furthermore, IL-6 induction in sepsis of mice seems to be neutrophil- and complement-dependent, since expression of the receptor for the complement protein C5a (C5aR) is required for induction of IL-6 (*149*). This hints to another layer of cellular interaction influencing the outcome of sepsis, possibly related to IL-6 induction in monocytes and their role in neutrophil recruitment.

In summary, it is plausible that monocytes and granulocytes release cytokines in a co-dependent manner to orchestrate the adaptive immune response to *S. suis*. The individual contributions of monocytes and granulocytes to the porcine cytokine response could be further elucidated by more elaborate cell separations and analyses, including transcriptional profiling, extensive surface marker analysis, and possibly by comparing monocytes from different stages of an acute *S. suis* infection.

4.3. **Outlook**

Since the uncontrolled induction of pro-inflammatory mediators like TNF-α or IL-6 seems to be associated with a detrimental outcome of invasive porcine *S. suis* infection, the regulation of those cytokines could be a potential target to treat *S. suis* infections.

The cell culture systems described here can be used in the future to investigate the relationship between *S. suis* infection and dysregulation of pro-inflammatory cytokines more closely. This system can not only be used to investigate the cytokine response and the cellular sources of the

dysregulated cytokines in more detail, but also to expand the system by comparing with cell culture systems using primary cells of secondary lymphoid organs, like lymph nodes and tonsil.

Other possibilities would be to investigate a possible relationship between the levels of *S. suis*-specific antibodies and pro-inflammatory cytokines, or an interaction between activated pro-inflammatory cells of the innate immune system and a corresponding immune response by lymphoid cells.

5. SUMMARY

Streptococcus suis is a common commensal in pigs and colonizes the mucosa and secondary lymphoid organs in healthy individuals. Invasive infections occur regularly in young weaning piglets (7-10 weeks of age), which cannot be fully explained by the immunological gap, a phase of deficiency in adaptive humoral immunity, or by highly virulent strains. Since virulent strains colonize the entire herd and the immunological gap occurs in all piglets at the same time, it is not clear why only some individuals are prone to *S. suis*-induced disease. *S. suis* first enters the blood (causing bacteremia) and subsequently spreads to peripheral organs, like brain, heart or joints (causing septic shock, meningitis, myocarditis or arthritis), which can be fatal. The transition from the colonizing state at the mucosa to invasive state in peripheral organs therefore has a key role in pathogenesis, but is still poorly understood. Since the systemic spread of *S. suis* occurs via the blood, the blood represents a critical stage before the bacteria can infect organs. It is incompletely understood why in some individuals, *S. suis* leads to controlled bacteremia, and in others the bacteremia evolves into an uncontrolled septicemia (or into septic/toxic shock). In humans, septicemia is defined as uncontrolled bacterial infection which is accompanied by a systemic inflammation, usually caused by bacterial surface components or secreted toxins. This systemic inflammatory reaction is caused by pro-inflammatory cytokines which are secreted by cells of the innate and adaptive immune system, and can amplify each other. It is questionable whether pathogenesis in septicemic pigs is comparable to *S. suis*-induced septic/toxic shock in humans. A number of infection studies in human cell lines and porcine whole blood demonstrated that the bacterial capsule inhibits phagocytosis efficiently, while immunomodulatory effects are mainly mediated by the bacterial cell wall. However, it remained unclear, if the situation *in vivo* in pigs is comparable. The aim of this study was to establish an *in vitro* system of primary porcine cells and to compare it to the cytokine response in pigs in an *in vivo* infection.

Some of the investigated infected pigs, which had a pronounced bacteremia 13 hours after infection, showed elevated levels of the pro-inflammatory cytokine IL-6 after 13 hours and also increased levels of the anti-inflammatory cytokine IL-10 after 16 hours of infection. However, the pro-inflammatory cytokines TNF-α, IFN-γ, or IL-17A were not elevated at these time points. TNF-α is typically induced early after an infection and was possibly already downregulated at the time of the earliest sampling (13 hours after infection). These pigs with a pronounced bacteremia showed a typical pro-inflammatory cytokine response, which is counteracted by anti-inflammatory cytokines at the end. On the other hand, pigs with controlled bacteremia did not develop blood levels of IL-6 and IL-10. In the subsequent analysis looking at *S. suis*-infected whole blood it was shown that the encapsulated wild type strain 10 already induced elevated TNF-α, IL-6 and IL-10 levels after 2 hours, but was not killed. The isogenic acapsular deletion mutant strain 10cpsΔEF induced a similar increase of cytokines at a comparable kinetic, but was killed

effectively. Furthermore, *in vitro*-infected peripheral blood mononuclear cells (PBMC) showed a higher production of TNF-α, IL-6 and even IL-10 when infected with acapsular *S. suis* compared to encapsulated wild type *S. suis*. Also, the cytokine levels were associated with the amount of *S. suis* present. Taken together, this allows the conclusion that encapsulated strains, which survive better, induce cytokines in lower amounts over a longer time. Conversely, acapsular strains seem to induce stronger cytokine responses, but are killed faster. Strains with different capsule compositions (*cps2*, *cps7*, *cps9*) showed no differences in cytokine induction indicating serotype-independent cytokine regulation. Additionally, it was shown that inhibition of endogenous IL-10 via a neutralizing antibody was able to limit release of TNF-α and IL-6. This confirms the previously mentioned feedback loop of anti-inflammatory cytokines regulating the pro-inflammatory response. For the understanding of dysregulated systemic cytokine secretion, the identification of the cellular sources of the secreted cytokines is essential. Therefore, monocytes and granulocytes were isolated from porcine PBMC and stimulated separately with *S. suis*. It could be shown that encapsulated *S. suis* induces TNF-α production mainly by monocytes, but not by granulocytes. The cellular sources of IL-6 and IL-10 could not be clearly identified. TNF-α is typically produced by monocytes and macrophages as a fast pro-inflammatory response upon infection and therefore it plays a crucial role in the development of sepsis. In this study it could be shown that TNF-α was released fast and in large amounts by monocytes. Therefore, the final question was whether TNF-α has a bactericidal effect during infection of porcine whole blood *in vitro*. Since antibodies in blood play an essential role in the killing of *S. suis*, the bactericidal effect of TNF-α was investigated in presence of high, low and especially in absence of *S. suis*-specific IgG. In presence of high *S. suis*-specific IgG, *S. suis* strain 10 was effectively killed, as expected. In the presence of low *S. suis*-specific IgG, recombinant TNF-α was also added or alternatively inhibited by a neutralizing antibody. Both treatments had no effect on the killing of *S. suis*. Even in the absence of *S. suis*-specific IgG, TNF-α did not affect bacterial survival. This suggests that TNF-α does not contribute to bacterial killing (even not in the complete absence of antibodies) in this experimental *in vitro* system, but that *S. suis*-specific antibodies are critical for rapid bacterial killing.

In summary, it could be shown that the pro- and anti-inflammatory cytokine induction in the *in vitro* whole blood model is comparable to the *in vivo* situation. The presence, but not the composition, of the bacterial capsule has a limiting impact on the cytokine induction, and also reduces the killing of bacteria, as shown previously. Monocytes could be identified as the main producers of TNF-α in porcine blood. Therefore, the *in vitro* model has contributed to the understanding of the induction of pro- and anti-inflammatory cytokines during *S. suis* infection of the blood. This model offers a basis for further investigation of the interaction and of the cellular sources of cytokines during infection of the blood by *S. suis*.

6. ZUSAMMENFASSUNG

Streptococcus suis ist ein weitverbreiteter Kommensale im Schwein, der im gesunden Tier asymptomatisch die Schleimhäute und sekundäre lymphatische Organe kolonisiert. In jungen Absatzferkeln von 7-10 Wochen Alter kommt es in Schweinebeständen regelmäßig zu invasiven Infektionen mit *S. suis*, die durch die immunologische Lücke, eine vorübergehend fehlende adaptive humorale Immunität der Ferkel zu dieser Zeit, sowie durch einzelne besonders virulente Stämme nur teilweise erklärbar ist. Da virulente Stämme in einer Herde jedoch üblicherweise weit verbreitet sind und die immunologische Lücke bei den Tieren gleichzeitig auftritt, ist es unklar, warum nur ein Teil der Schweine vom Ausbruch der Krankheit betroffen ist. *S. suis* breitet sich während eines Ausbruchs im Blut aus (Bakteriämie bzw. Septikämie) und infiziert darüber periphere Organe wie das Gehirn, das Herz, sowie Gelenke, was zu septischem Schock, Meningitis, Myokarditis oder Arthritis und schließlich zum Tod führen kann. Der Übergang vom kolonisierenden Stadium auf den Schleimhäuten zum invasiven Stadium in den peripheren Organen spielt demzufolge eine Schlüsselrolle in der Krankheitsentwicklung, ist bisher jedoch schlecht verstanden. Da die Verbreitung von *S. suis* im Organismus über das Blut erfolgt, ist das Blut die letzte Hürde, bevor *S. suis* peripheren Organe infiziert. Es ist bisher unklar, warum nur in einigen Individuen *S. suis* invasiv wird, und nur in einigen Schweinen aus einer Bakteriämie eine unkontrollierte Septikämie bzw. septischer Schock entsteht. Bei Menschen spricht man von einer Septikämie, sobald eine bakterielle Infektion nicht mehr kontrolliert werden kann, sondern durch bakterielle Oberflächenmoleküle oder freigesetzte Toxine eine systemische Entzündungsreaktion ausgelöst wird. Diese Entzündungsreaktion ist auf unkontrolliert ausgeschüttete pro-inflammatorische Zytokine durch Zellen des angeborenen und adaptiven Immunsystems zurückzuführen, die sich gegenseitig verstärken. Es ist fraglich, ob die Krankheitsentstehung in septikämischen Schweinen vergleichbar mit der Situation im Menschen ist. Durch einige Studien von *S. suis*-Infektion in menschlichen Zelllinien und porzinem Vollblut ist bekannt, dass die bakterielle Kapsel effektiv Phagozytose verhindert während immunmodulatorische Effekte hauptsächlich von der bakteriellen Zellwand vermittelt werden, doch die Vergleichbarkeit mit der Situation im Schwein *in vivo* ist unklar. Ziel der Arbeit war es daher, ein *in vitro*-System mit primären Schweinezellen zu etablieren und mit der Zytokinantwort *in vivo* in Schweinen zu vergleichen.

In einigen der *in vivo*-infizierten Schweine, die 13 Stunden nach Infektion mit *S. suis* Stamm 10 eine ausgeprägte Bakteriämie zeigten, waren nach 13 Stunden bereits das pro-inflammatorische Zytokin IL-6 sowie nach 16 Stunden das anti-inflammatorische Zytokin IL-10 erhöht, nicht jedoch die pro-inflammatorischen Zytokine TNF-α, IFN-γ oder IL-17A. TNF-α ist typischerweise sehr früh nach einer Infektion induziert und war zum Zeitpunkt der frühesten Probennahme (13 h nach Infektion) möglicherweise wieder abgeklungen. Dieses Bild entspricht einer typischen pro-

inflammatorischen Zytokinantwort, in deren Anschluss anti-inflammatorische Zytokine produziert werden, um die Entzündung auszubalancieren. Diejenigen Schweine, die eine Bakteriämie kontrollieren konnten, zeigten auch keine erhöhten Mengen an IL-6 oder IL-10. In einer weiteren Analyse mit *S. suis*-infiziertem Vollblut zeigte sich, dass der bekapselte Wildtypstamm 10 nach 2 Stunden bereits erhöht TNF-α, IL-6 sowie IL-10 induzierte, jedoch nicht abgetötet wurde. Die unbekapselte Deletionsmutante 10cpsΔEF induzierte dieselben Zytokine in einer vergleichbaren Kinetik, wurde jedoch erwartungsgemäß abgetötet. Weiterhin zeigten *in vitro* infizierte mononukleäre Zellen des peripheren Bluts (PBMCs) bei Infektion mit unbekapseltem *S. suis* eine höhere Produktion von TNF-α und IL-6 aber auch von IL-10 im Vergleich zu bekapseltem Wildtyp. Darüber hinaus war die Zytokinmenge abhängig von der *S. suis*-Infektionsdosis. Zusammengenommen lässt dies darauf schließen, dass die besser überlebenden bekapselten Stämme Zytokine in geringerer Menge, aber dafür über einen längeren Zeitraum induzieren. Umgekehrt scheinen unbekapselte Stämme Zytokine stärker zu induzieren, aber auch schneller abgetötet zu werden. Stämme mit verschiedenen Kapselgenen (*cps2*, *cps7*, *cps9*) zeigten jedoch keine Unterschiede in der Zytokininduktion, was auf eine Serotyp-unabhängige Zytokinregualation hinweist. Darüber hinaus konnte in Zellkultur von PBMCs gezeigt werden, dass die Neutralisation von endogenem IL-10 die Induktion von TNF-α und IL-6 limitiert, was wieder die zuvor gezeigte Selbstregulation der freigesetzten pro-inflammatorischen Zytokine in den infizierten Schweinen *in vivo* bestätigt. Zum Verständnis einer dysregulierten Zytokinausschüttung ist die Identifizierung der Zytokin-produzierenden Zellen entscheidend. Deshalb wurden Monozyten und Granulozyten aus PBMCs des Schweins isoliert und separat mit *S. suis* infiziert. Dabei konnte bestätigt werden, dass bekapselte *S. suis* TNF-α hauptsächlich von Monozyten produziert wird, nicht von Granulozyten. Für IL-6 und IL-10 konnten die Produzenten nicht eindeutig zugeordnet werden. TNF-α wird typischerweise von Monozyten und Makrophagen als schnelle pro-inflammatorische Reaktion auf Infektionen freigesetzt und spielt eine entscheidende Rolle in der Entwicklung einer Sepsis. Auch in den Untersuchungen dieser Arbeit konnte gezeigt werden, dass TNF-α nach Infektion mit *S. suis* schnell und in großen Mengen von Monozyten freigesetzt wird. Daher sollte schließlich *in vitro* in porzinem Vollblut untersucht werden, ob TNF-α eine bakterizide Wirkung hat. Da im Blut Antikörper eine entscheidende Rolle bei der Abtötung von *S. suis* spielen, wurde der bakterizide Effekt von TNF-α in Gegenwart von unterschiedlich hohen Spiegeln und besonders in Abwesenheit von *S. suis*-spezifischem IgG untersucht. In Gegenwart eines hohen IgG-Spiegels wurde *S. suis* wie erwartet effektiv abgetötet. In Gegenwart von niedrigem IgG-Spiegel und in Abwesenheit von IgG wurde rekombinantes TNF-α zusätzlich zugegeben bzw. durch einen neutralisierenden Antikörper blockiert. Beide Behandlungen hatten keinerlei Einfluss auf das Abtöten der Bakterien. Dies deutet darauf hin, dass TNF-α in diesem *in vitro*-System nicht zum Abtöten der Bakterien beiträgt (auch nicht in

Abwesenheit von Antikörpern), sondern *S. suis*-spezifische Antikörper entscheidend für eine schnelle bakterielle Abtötung sind.

Zusammenfassend konnten gezeigt werden, dass pro- und anti-inflammatorische Zytokine im *in vitro*-Vollblutmodell und im Schwein vergleichbar induziert werden, und dass das Vorhandensein und nicht die Komposition der bakteriellen Kapsel einen limitierenden Einfluss auf die Zytokin-Induktion und auf das Abtöten der Bakterien hat, wie in vorangegangenen Studien bereits gezeigt wurde. Monozyten konnten als Hauptproduzenten von TNF-α im Blut des Schweins identifiziert werden. Damit hat das *in vitro*-Modell zum Verständnis der Induktion von pro- und anti-inflammatorsichen Zytokinen während der Infektion des Blutes mit *S. suis* beigetragen. Damit bietet es eine Grundlage für die weitere Erforschung der Interaktion und zellulären Quellen von Zytokinen während einer *S. suis*-Infektion des Blutes.

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8. ACKNOWLEDGMENT

9. SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel: „Analysis of porcine cytokine response upon *Streptococcus suis* infection *in vivo*, in whole blood and in cell culture" selbstständig und ausschließlich unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe.

Leipzig, _____   _____

                                                                                    Florian Hohnstein

10. WISSENSCHAFTLICHER WERDEGANG

Personal Data

Name	Florian S. Hohnstein
Born	04. January 1991 in Leipzig
Nationality	German
Email	fs.hohnstein@gmail.com

School and Academic Education

04/2016 – present — PhD student at the Institute of Immunology, Faculty of Veterinary Medicine, Universität Leipzig

- PhD thesis: „Analysis of porcine cytokine response upon Streptococcus suis infection in vivo, in whole blood and in cell culture"

10/2013 – 12/2015 — Master of Science in Molecular Cell Biology & Immunology, University of Tübingen

- Master thesis: „Generating recombinant Orf virus for the induction of an *in vitro* immune response"

10/2010 – 09/2013 — Bachelor of Science in Biology, University of Tübingen

- Bachelor thesis: „The role of the Guanine nucleotide exchange factor C3G during ventral furrow formation in *Drosophila melanogaster*"

08/2001 - 06/2009 — Abitur, Neue Nikolaischule, Leipzig

Working experience and Methods

04/2016 – 09/2019 — Research associate at the Institute of Immunology, Faculty of Veterinary Medicine, University of Leipzig

- Methods: Flow cytometry, primary cell culture, ELISA, bacterial isolation

04/2015 – 09/2015 — Research assistant at the Institute of Immunology, University of Tübingen

- Methods: virus culture, virus titration, bacterial transformation, cell transfection, molecular cloning, genetic engineering (CRISPR/Cas9), primary cell culture of dendritic cells and T cells, culture of Vero cells, flow cytometry

12/2014   Student assistant at the Institute of Cell Biology, Department of Animal Genetics, University of Tübingen

- Methods: DNA isolation, PCR, cross-breeding genetics, confocal laser scanning microscopy, live imaging, digital image processing, *in silico* analysis of protein homology, practical fly work

11/2011 – 03/2013   Student assistant at the Department of Molecular Biology, Max Planck Institute for Developmental Biology

- Methods: DNA isolation, PCR, bacterial transformation, practical plant work

Qualifications

03/2015   Nachweis von Kenntnissen und Fähigkeiten gemäß Anlage 1 Abschnitt 1 bis 3 TierSchVersV (officially approved)

Leipzig, _____   _____

Florian Hohnstein

11. PUBLIKATIONSLISTE

Parts of this work were published in a peer-reviewed scientific journal:

1. <u>Hohnstein FS</u>, Meurer M, de Buhr N, von Köckritz-Blickwede M, Baums CG, Alber G, Schütze N, "Analysis of Porcine Pro- and Anti-Inflammatory Cytokine Induction by *S. suis In Vivo* and *In Vitro*", Pathogens 2020, 9, 40; doi:10.3390/pathogens9010040. Chosen as cover story for this Special Issue on *Streptococcus suis*.

Posters

2. Schütze N, <u>Hohnstein FS</u>, Rungelrath V, Büttner M, Baums CG, Alber G, "Innate TNF-α and IFN-γ production induced by *Streptococcus suis* in procine PBMC is dependent on monocyte activation", 6th European Veterinary Immunology Workshop (2018), Utrecht, Netherlands.

3. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "Analysis of the *Streptococcus suis*-induced cytokine profile in porcine PBMCs and blood", Novel Concepts of Innate Immunity (2019), Tübingen, Germany.

4. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "*In vitro* analysis of the *Streptococcus suis*-induced cytokines in porcine PBMCs and blood", 15th Research Festival for Life Sciences (2019), Leipzig, Germany.

5. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "*In vitro* analysis of the *Streptococcus suis*-induced cytokines in porcine PBMCs and blood", 7. Leipziger Doktorandenforum (2019), Leipzig, Germany.

6. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "*In vitro* analysis of the *Streptococcus suis*-induced cytokine milieu in porcine peripheral blood mononuclear and tonsil cells", 14th Research Festival for Life Sciences (2018), Leipzig, Germany.

7. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "*In vitro* analysis of the *S. suis*-induced cytokine milieu during induction of the porcine cellular immune response", 6. Leipziger Doktorandenforum (2018), Leipzig, Germany.

8. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, Analysis of functional T-cell subsets in porcine blood and secondary lymphoid organs with a potential role in immunity against *Streptococcus suis* infection", Annual meeting of the AK veterinary immunology of the DGfI (2017), Bern, Switzerland.

9. <u>Hohnstein FS</u>, "Analysis of cell-mediated immunity upon infection with *Streptococcus suis* in pigs", 5. Leipziger Doktorandenforum (2017), Leipzig, Germany.

10. <u>Hohnstein FS</u>, Baums CG, Alber G, Schütze N, "Analysing cell-mediated immunity in pigs upon infection with *Streptococcus suis"*, 8th Autumn School of the German Society for Immunology e.V. (DGfI) (2016), Merseburg, Germany.

Presentations

11. <u>Hohnstein FS</u>, "*In vitro* analysis of the *Streptococcus suis*-induced cytokines in porcine PBMCs", Mini symposium "Infection and Immune defense" (2019), Rothenfels, Germany.

12. <u>Hohnstein FS</u>, "T-cell response to *Streptococcus suis*", Doktoranden-Kolloquium des BBZ (2018), Leipzig, Germany.

13. <u>Hohnstein FS</u>, "Analysis of cell-mediated immunity in pigs upon infection with *Streptococcus suis*", Doktoranden-Kolloquium des BBZ (2017), Leipzig, Germany.