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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

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

Unless otherwise indicated, S. suis strains were cultured in Todd-Hewitt broth (THB, Becton Dickinson, 249240) or on Columbia agar plates with 5% sheep blood (Oxoid, PB5039A) at 37°C and 5% CO2. Escherichia coli (E. coli) DH5α was grown in lysogeny broth (LB, Carl Roth, X968.1) at 37°C under constant shaking or on LB agar plates. When needed, chloramphenicol (Cm, Carl Roth, 3886.2) was added at a concentration of 3.5µg/ml for S. suis and 8µg/ml for E. coli.

DNA Techniques and Primers

Restriction enzymes (BamHI R0136, Sall R0138, PstI R0140, CIP M0525, and AccI R0161), ligase (ElectroLigase® M0369), and polymerases (OneTaq® M0480, Phusion® M0530) were purchased from New England Biolabs and used according to manufacturer’s recommendations. Standard DNA manipulations were performed as described (Green and Sambrook, 2012). Chromosomal DNA of S. suis strain 10 served as a template for all PCRs conducted for generation of inserts. Oligonucleotide primers were designed based on
the sequence of SSU0596 in the genome of S. suis P1/7.1 This nucleotide sequence is also found without any difference in the recently published genome of strain 10 (SSU10_RS03070; Bunk et al., 2021) as well as in the 2020 published genome of S10 (van der Putten et al., 2020).

Targeted Mutagenesis of dltA in Streptococcus suis Strain 10 and 10cpsΔEF
The thermosensitive shuttle vector pSET5s (Takamatsu et al., 2001) was used as a backbone to generate the plasmid pSET5ΔdltA enabling allelic exchange of dltA as follows:

DNA fragments corresponding to regions upstream and downstream of the dltA gene were amplified using primers dltA_FrA_for_BamHI (TAAGGATCCCTCATT TTTTGGCA ATG) and dltA_FrA_rev_Sall (ATGTTGACGGCTCTTAAC ATACTGCTA) as well as dltA_FrB_for_Sall (TCCGTCGACTG CAAATGGGAAGTTT) and dltA_FrB_rev_PstI (GAT CTTGACGGCCACCTGGAATTTG). The fragments were digested with the restriction enzymes indicated in the names of the primers and inserted in the multiple cloning site of pSET5s.

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

The isogenic mutant 10ΔdltA was verified by PCR, sequencing of the deletion site and non-radioactive Southern blot analysis as described before (Rungelrath et al., 2018). Chromosomal DNA was digested by AccI and primer pairs used for generation of the probes were dltA_Suis_for (5’-ATGTTGAGGGTCCGTA CGCTTG-3’) plus dltA_Suis_rev (5’-AAGTTGGCCAGTGCTG TGTTTGG-3’) to verify the absence of the dltA gene and pSET5sSondefor (5’-CGAAAAAAAGGATTATGATTTCTCTG TTTTGG-3’) plus pSET5sSonderev (5’-GGTTTTTTATAGTGCTT GCTCA TTTTGG-3’) to confirm complete excision of the plasmid from the bacterial chromosome.

To construct a 10cpsΔEFΔdltA double mutant, we transformed the plasmid pSET5ΔdltA into the 10cpsΔEF mutant and followed the mutagenesis protocol described above.

Extraction and Isolation of LTA
Bacteria were grown in THB to an optical density at 600 nm (OD600) of 1 and harvested by centrifugation at 5,000 × g, 4°C for 15 min. LTA isolation and purification were performed as described elsewhere (Heß et al., 2017). Yields of LTA preparations from 5 L of bacterial culture were as follows: strain 10, 8.7 mg; strain 10ΔdltA, 3.9 mg.

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

Extraction of mRNA and qRT-PCR
Bacterial RNA was extracted from exponential-phase THB cultures (OD600 of 0.5) of the different S. suis strains used in this study. In detail, the pellet of a 10-ml culture (centrifugation: 10 min, 2,600 × g, 4°C) was resuspended in 1 ml ice-cold Trizol (Sigma T9424) and stored at −80°C until RNA extraction. Isolation was conducted as previously described (Willenborg et al., 2011). Concentration and purity of the isolated RNA were determined using the Agilent 2100 bioanalyzer (RNA 6000 Pico kit; Agilent Technologies Inc., Santa Clara, CA, United States). Quantitative real-time PCR (qRT-PCR) of reverse-transcribed RNA was designed to analyze the expression of dltA, dltC—dltD, SSU10_RS03090 (low temperature requirement protein A, downstream of dltD), SSU10_RS03040 (glucosamin-6-phosphate deaminase, upstream of dltA), and the housekeeping gene gyrB. The respective primers are listed in Supplementary Table S1. qRT-PCR was conducted with the AriaMX real-time PCR system (Agilent Technologies Inc., Santa Clara, CA, United States), as previously described (Willenborg et al., 2011). The following modified program was used as: an initial denaturation step at 95°C for 15 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and amplification at 72°C for 30 s. As negative controls, we included (i) water, (ii) a no-template control, and (iii) no reverse transcriptase control to exclude false positive results due to contamination with DNA. Products were verified by melting-curve analysis and 1.5% agarose gel electrophoresis.

Cytochrome C Binding Assay
The assay was essentially performed as previously described (Kristian et al., 2005), except that bacteria were adjusted to an OD600 of 1 in morpholinepropanesulfonic acid (MOPS) buffer (20 mM, pH 7) before adding 0.5 mg/ml cytochrome C for 10 min at room temperature. After centrifugation (14,000 × g, 3 min, room temperature), the cytochrome C content of the supernatant was quantified photometrically at 530 nm (OD530). MOPS buffer containing 0.5 mg/ml cytochrome C was incubated under the same conditions without bacteria as a control (OD0). The percentage of bound cytochrome C was calculated as follows:

\[
\text{Bound cytochrome C(%) = } \left( \frac{\text{OD}_A - \text{OD}_B}{\text{OD}_A} \right) \times 100
\]

Microbial Adhesion to Hydrocarbons Assay
Hydrophobicity of S. suis was evaluated by measuring bacterial adhesion to hexadecane (Sigma, H6703) following a previously described protocol (Srikham et al., 2021) with slight modifications.
Briefly, *S. suis* strains were cultured overnight and harvested by centrifugation (3,900×g, 10 min, 4°C). Pellets were resuspended in PBS and washed twice before adjusting the suspensions to an OD$_{600}$ of 1 (OD$_{A}$). Then, 2 mL of bacterial suspension was mixed with 400 μL of hexadecane and tubes were vortexed for 30 s. The mixture was allowed to separate into two phases for 30 min at room temperature. The aqueous phase was collected and OD$_{600}$ (OD$_{A}$) was measured. Cell surface hydrophobicity was calculated as follows:

\[
\text{Hydrophobicity} = \left[1 - \left(\frac{\text{OD}_B}{\text{OD}_A}\right)\right] \times 100
\]

**Antimicrobial Susceptibility**

The cationic peptide PR-39 (RRPRPPPYLPRPRPPPFPPLLPRPFPFGPRFP) was kindly provided by Ralf Hoffmann from the Institute of Bioanalytical Chemistry of the University of Leipzig. Bacitracin was obtained from Carl Roth (5655.1), colistin, and polymyxin B were obtained from Sigma (C5561 and P4932). Minimal bactericidal concentrations (MBC) were defined as the lowest concentration of the antimicrobial agent where no bacterial growth occurred. To determine the MBC, we followed a previously described protocol for evaluation of minimal inhibitory concentrations (MIC; Fittipaldi et al., 2008) and additionally plated aliquots of the suspensions on blood agar plates after the incubation period of 24 h at 37°C. While *S. suis* was cultured in 100 μL THB with serial dilutions of bacitracin, colistin, and polymyxin B as described in the above-mentioned protocol, evaluation of PR-39 MBC was performed in 50 μL Rosewell Park Memorial Institute (RPMI) 1640 (Gibco, 11835063) containing 5% cation-adjusted Mueller Hinton broth (CA-MHB, Oxoid CM0405, MgCl$_2$·6H$_2$O Sigma M2670, and CaCl$_2$·2H$_2$O Merck 2382) based on the finding that MICs measured in this medium resembled MICs determined in cerebrospinal fluid of pigs (Meurer et al., 2019). Each assay was performed in triplicates and repeated at least three times.

**Bactericidal Assays in Whole Blood**

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

**Far Red Labeling of Streptococcus suis**

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

**Combined Oxidative Burst and Granulocyte Association Assay**

Measurement of oxidative burst and the association of *S. suis* with porcine granulocytes was essentially conducted as described before (Rungelrath et al., 2020), except that whole blood samples were used. Briefly, *S. suis* FR stocks were added to 100 μL whole blood of 8-week-old piglets at a concentration of 10$^7$ CFU/ml. After 15 min of incubation at 37°C, dihydrorhodamine123 (DHR123, Sigma, D1054) was added to stain reactive oxygen species (ROS) within the granulocytes. While reacting with ROS, DHR123 is oxidized to fluorescent rhodamine123 (Rho123). Samples were measured by flow cytometry (BD FACSCalibur) and analyzed with FlowJo™ V10 software.

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

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by a density gradient separation as described previously (Hohnstein et al., 2020). PBMCs (10$^7$ cells/ml) were infected with *S. suis* FR at an MOI of 1 for 30 min at 37°C, whereby *S. suis* had been pre-incubated in porcine serum of colostrum-deprived piglets (CDS) or in hyperimmune serum #4515 generated through prime-boost vaccination of a weaning piglet with a strain 10 bacterin (both obtained within previous studies; Weifie et al., 2021). Monocytes were stained using the myeloid marker CD172a-FITCs (BD Pharmingen™, 561498, 0.5 mg/ml). Lymphocytes were defined as CD172a-negative PBMCs. Samples were measured by flow cytometry (BD FACSCalibur) and analyzed with FlowJo™ V10 software. Following incubation with bacteria for 30 min or 2 h, in a subset of samples, PBMCs were stained with viability dye eFluor™ 506 (Thermo Fisher Scientific, 65-0866-14) according to manufacturer’s recommendations. These samples were measured using BD FACS Fortessa.

**Cytospin and Staining for Optical Microscopy**

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

**Cytokine Quantification**

DuoSet ELISA kits for porcine tumor necrosis factor (TNF)-α and IL-1β were purchased from R&D Systems (DY690B and DY681) and performed essentially according to manufacturer’s recommendations. The analysis was conducted with supernatants or plasma obtained before and after infection with *S. suis* in...
the d-alanine-d-alanyl carrier protein ligase in order to investigate the role of d-alanlylation of LTAs in pathogen-host interaction. To verify the absence of alanine residues in the LTAs of \( S. \) \( suis \) 10\( \Delta \)dltA, LTA was isolated from late exponential wt and mutant cultures and subjected to NMR spectroscopy. Overall, the \( ^1 \)H NMR spectrum recorded for LTA isolated from the wt (Supplementary Figure S1, top panel) is virtually identical with those obtained earlier for LTA isolated from other \( S. \) \( suis \) serotype 2 strains (strains P1/7 and SC84; Gisch et al., 2018) and further NMR analysis confirmed the identical overall structure. The peaks indicative for d-alanine residues present at the position O-2 of glycerol within the poly-(glyco) glycerolphosphate chain \( [\delta_1 \text{H}, 1.65–1.60 \text{ (Ala-CH)}] \), 4.33–4.27 (Ala-CH), and 5.42–5.36 (glycerol-H2) ppm; Fittipaldi et al., 2008; Gisch et al., 2018) were absent in the spectra recorded from LTA of the \( \Delta \)dltA strain (Supplementary Figure S1). This result confirms that the dltA gene is necessary for d-alanlylation of LTA in \( S. \) \( suis \), as already described previously (Fittipaldi et al., 2008).

**mRNA Expression Analysis of the dlt Operon and Adjacent Genes**

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

**Effects of LTA d-Alanlylation on Surface Charge, Hydrophobicity, and Susceptibility to Cationic Antimicrobial Peptides**

We hypothesized in agreement with results for Group A streptococci (Kristian et al., 2005) and pneumococci (Saar-Dover et al., 2012) that the net negative charge of the bacteria is increased in the two \( \Delta \)dltA mutants. However, we found no significant difference in binding of the cationic peptide cytochrome C between \( S. \) \( suis \) wt and 10\( \Delta \)dltA (Figure 1A; strain 10: mean 38.27%, SD 4.43%; 10\( \Delta \)dltA: mean 34.99%, 95% was chosen for all analyzes. All figures and data in statistical analysis were performed using BD FACS Fortessa and analyzed using FlowJo™ V10 software.

**Statistical Analysis**

Statistical analysis was performed using Prism software, version 9 (GraphPad, San Diego, CA, United States). Normality was tested by Shapiro–Wilk test. Differences between multiple groups or time points were determined using ANOVA followed by Holm-Sidak’s or Tukeys multiple comparisons test, respectively. Differences between wt and dltA mutant were tested using two-tailed paired t-test or Wilcoxon test when appropriate. Treatments with the two different porcine sera were compared by two-tailed unpaired t-test or Mann–Whitney test. A CI of 95% was chosen for all analyzes. All figures and data in parentheses in the text represent the means and SD. Probabilities were considered as indicated in the figure legends.

**RESULTS**

**Deletion of the dltA Gene Abolishes d-Alanlylation of Streptococcus suis LTA**

We created an isogenic mutant of the virulent serotype 2 strain 10 by in-frame deletion of the dltA gene, encoding for

PBMC samples or bactericidal assays in whole blood as described above. The streptavidin-horseradish peroxidase used to couple the detection antibodies was detected with a 3,3′,5,5′-Tetramethylbenzoin (TMB) solution (SeraCare, Milford, MA, United States, formerly KPL) and the reaction was stopped after 20min with 1M \( \text{H}_2\text{PO}_4 \) (Roth, 6366.1). OD values were measured with a microplate reader SpectraMax 340PC384 (Molecular Devices, LLC San Jose, CA, United States) at 450 and 630nm as a background reference and analyzed with SoftMax® Pro v5.0 software (Molecular Devices, LLC; Hohnstein et al., 2020).

**C3 Deposition on the Surface of Streptococcus suis**

Deposition of complement on the streptococcal surface was measured essentially as described before (Rungezahl et al., 2018). Briefly, \( S. \) \( suis \) strains were grown to an OD\(_{600}\) of 0.5 in THB. Then, 50μl of the respective culture was incubated with 100μl of CDS for 1h at 37°C under rotation (8rpm). As negative control, CDS was incubated for 30 min at 56°C to inactivate all complement factors. Staining of C3 labeled bacteria was conducted with 200μl of a 1:150 diluted FITC-labeled cross-reactive rabbit anti-human C3 antibody (Dako, F02102-2, 3g/L) for 1h at 4°C. Samples were measured using BD FACS Fortessa and analyzed using FlowJo™V10 software.

**Anti-Streptococcus suis IgG and IgM ELISA**

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

**Statistical Analysis**

Statistical analysis was performed using Prism software, version 9 (GraphPad, San Diego, CA, United States). Normality was tested by Shapiro–Wilk test. Differences between multiple groups or time points were determined using ANOVA followed by Holm-Sidak’s or Tukeys multiple comparisons test, respectively. Differences between wt and dltA mutant were tested using two-tailed paired t-test or Wilcoxon test when appropriate. Treatments with the two different porcine sera were compared by two-tailed unpaired t-test or Mann–Whitney test. A CI of 95% was chosen for all analyzes. All figures and data in parentheses in the text represent the means and SD. Probabilities were considered as indicated in the figure legends.
SD 3.46%). In contrast, loss of LTA d-alanylation in the non-encapsulated mutant caused a highly significant increase in bound cytochrome C (10cpsΔdltA: mean 25.57%, SD 2.32%; 10cpsΔEFΔdltA: mean 33.25%, SD 2.76%). These results suggest that changes in local charges due to LTA d-alanylation in the wt bacteria are masked by the capsular polysaccharides.

We further investigated hydrophobicity of the different streptococci by the microbial adhesion to hydrocarbons assay. While the capsule provided a hydrophilic character to the streptococcal surface and its absence caused a strongly significant increase of hydrophobicity (strain 10: mean 22.18%, SD 3.66%; 10cpsΔEF: mean 82.60%, SD 5.10%); 10ΔdltA and 10cpsΔEFΔdltA showed a significantly reduced cell surface hydrophobicity compared to the wt and the capsular mutant 10cpsΔEF, respectively (10ΔdltA: mean 12.39%, SD 6.08%; 10cpsΔEFΔdltA: mean 73.24%, SD 8.80%; Figure 1B). In conclusion, LTA d-alanylation increases cell surface hydrophobicity of encapsulated as well as non-encapsulated S. suis.

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

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

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

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

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

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

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

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

In the absence of specific antibodies (CDS-treated bacteria), LTA d-alanylation significantly reduced the level of association of S. suis with monocytes (Figure 3B, left graph, CDS; strain 10: mean 8.78%, SD 2.95%; 10ΔdltA: mean 12.63%, SD 3.6%), while the association was strongly enhanced for both strains when bacteria were opsonized with specific antibodies (Figure 3B, left graph, hyperimmune serum; strain 10: mean 36.41%, SD 6.43%; 10ΔdltA: mean 39.87%, SD 5.48%). In these hyperimmune conditions, the influence of LTA d-alanylation on the association of S. suis with monocytes remained significant but less pronounced. In microscopic analysis, the association
of monocytes with CDS-treated S. suis resembled a binding of the streptococci to the cell surface, whereas opsonization with hyperimmune serum partly resulted in streptococci visible within the membrane borders of monocytes, indicating a possible uptake by phagocytosis (Figure 3C).

As the capsule of S. suis strongly influences its interaction with mononuclear cells (Smith et al., 1999; Meijerink et al., 2012), we repeated the assays using the non-encapsulated mutant 10cpsΔEF and the double mutant 10cpsΔEFΔdltA to exclude the influence of capsular polysaccharides on the dltA phenotype.
Even in the absence of specific antibodies, the non-encapsulated mutants showed a very high level of association with monocytes (Figure 3B, right graph, 10cpsΔEF: mean 68.59%, SD 4.99%) and the loss of LTA d-alanylation significantly increased the number of FR positive monocytes (10cpsΔEFΔdltA: mean 71.76%, SD 5.10%), similar to what was observed for the encapsulated strains. These results indicate that d-alanylation of LTA modulates association of S. suis with monocytes independently of the capsular polysaccharides.

When analyzing the association of the streptococci with lymphocytes contained in the PBMC samples (Figure 3D), we found that the bacteria also adhered to lymphocytes in a
FIGURE 3 | Far Red-labeled *Streptococcus suis* ΔdltA mutants show a significantly higher level of association with porcine monocytes and lymphocytes. Peripheral blood mononuclear cell (PBMCs; freshly isolated from porcine blood) were incubated with Far Red-labeled *S. suis* strains (*S. suis* FR) at an MOI of 1 for 30 min at 37°C, whereby *S. suis* FR had been pre-incubated in serum of colostrum-deprived piglets (CDS) or in hyperimmune serum for 30 min, as indicated. Monocytes were stained using the myeloid marker CD172a-FITCs (as visualized in Supplementary Figure S4) and samples were measured by flow cytometry. The gating strategy for monocytes (A) and lymphocytes (D) in association with *S. suis* is visualized for PBMCs of one animal, while (B; monocytes) and (E; lymphocytes) show the entire results for the encapsulated strains on the left and the non-encapsulated strains on the right. The number of symbols represents the number of piglets PBMCs were obtained from. (C) Shows exemplary pictures of CDS-treated *S. suis* bound to the surface of monocytes (arrow) and uptake of antibody-opsonized *S. suis* (#) into monocytes as visualized by optical microscopy (x400 magnification). Horizontal lines and error bars represent mean values and SDs. For statistical analysis, paired t-test (wt or 10cps ΔEF vs. respective ΔdltA mutant) or unpaired t-test (CDS vs. hyperimmune samples) were performed. In case of not normally distributed samples, Wilcoxon test or Mann–Whitney test was used. Differences that are not indicated are not significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
dltA-dependent manner when no specific antibodies where present, as CDS-treated 10ΔdltA showed a significantly increased association with lymphocytes (Figure 3E, left graph; CDS; strain 10: mean 6.73%, SD 2.53%; 10ΔdltA: mean 8.74%, SD 3.28%). The same was visible in the non-encapsulated strains, although the increase was small (Figure 3E, right graph; 10cpsΔEF: mean 11.48%, SD 1.81%; 10cpsΔEFΔdltA: mean 12.39%, SD 1.76%). In contrast to monocytes, the increase of lymphocytes associated with S. suis due to opsonization with specific antibodies was far less pronounced (Figure 3E, left graph, hyperimmune serum; strain 10: mean 11.68%, SD 6.04%; 10ΔdltA: mean 12.76%, SD 7.24%) and an influence of LTA α-alanylation was no longer detected.

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

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

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

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

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

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

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

Purified LTAs obtained from strain 10 and 10ΔdltA were applied to porcine blood (final LTA concentration 30 µg/ml), which was subsequently incubated for 2 and 6 h. The concentrations of TNF-α and IL-1β after 2 and 6 h varied between samples but did not seem to depend on the dltA genotype of the original strain. Of note, IL-1β levels were below the limit of detection of 0.032 ng/ml after 2 h but above...
5 ng/ml (mean 15.53 and 18.80 ng/ml for LTAs of wt and $10\Delta dltA$, respectively) after 6 h indicating a delayed induction of IL-1β secretion by the purified LTAs in comparison with whole living $S.\ suis$ in this assay (Figure 6C).

In conclusion, infection of porcine blood $ex\ vivo$ with $S.\ suis$ wt and $10\Delta dltA$ indicates that $\beta$-alanylation of LTAs results in increased secretion of IL-1β, while the level of alanylation of purified LTAs, used for $ex\ vivo$ stimulation of
porcine blood, did not influence the amount of secreted IL-1β. Therefore, the reduction of IL-1β levels, observed in blood infected with the ΔdltA mutant, is most likely not attributable to a directly reduced activation of PRRs in porcine immune cells by the LTAs of 10ΔdltA compared to the LTAs of the wt.

**DISCUSSION**

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

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

**FIGURE 5** | Labeling with complement factor C3 is increased in Streptococcus suis 10ΔdltA after incubation in active CDS. Streptococcus suis strains were grown to early exponential phase (OD_{600} of 0.5) in THB and incubated with CDS for 1 h, followed by staining of C3 with a cross-reactive FITC conjugated anti-human C3c antibody and measurement of C3 associated with the bacterial surface by flow cytometry. (A) Represents the gating strategy and negative control sample, while (B; encapsulated S. suis) and (C; unencapsulated S. suis) visualize representative examples on the left and the complete data sets on the right. Horizontal lines and error bars represent mean values and SDs. For statistical analysis, unpaired t-test was used to compare parent strain and respective ΔdltA mutant. ***p < 0.001.
mutant. However, for the observed increase in resistance to CAMPs, it might not be crucial for \textit{S. suis} to increase its net surface charge. Saar-Dover et al. (2012) suggest that the anionicity of the bacterial surface does not modulate the accumulation of CAMPs and the increased resistance resulting from \(\beta\)-alanylation of Group B \textit{Streptococcus} LTA might rather be attributed to an altered conformation of the LTAs and resulting modulations in the density and surface properties of the bacterial cell wall. Therefore, even without increasing the global charge, LTA \(\beta\)-alanylation could contribute to CAMP resistance in \textit{S. suis}. In the absence of the capsule, we observed an effect of LTA \(\beta\)-alanylation on the electric charge of the bacterial surface as determined by cytochrome C binding. The non-encapsulated \(\Delta\text{dltA}\) mutant bound a significantly higher amount of cytochrome C than its non-encapsulated parent strain. This suggests that \(\beta\)-alanylation of LTA makes a difference for the electric charge of the bacterial surface if the capsule is downregulated and that capsular polysaccharides of \textit{S. suis}
can mask this electric charge. Furthermore, we found an increased surface hydrophobicity due to the introduction of \(\beta\)-alanine into LTAs of \emph{S. suis} as determined by the microbial adhesion to hydrocarbons assay. This might be caused by the hydrophobic character of the methyl group in alanine.

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

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

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

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

In difference to our finding of an augmented secretion of IL-1β and TNF-α in response to antibody-opsonized bacteria, Segura et al. (2006) described reduced mRNA expression of cytokines in a whole blood culture system with \emph{S. suis} opsonized by specific antibodies, possibly due to suppressed bacterial growth. In our PBMC samples, bacteria proliferated even in the presence of high levels of specific antibodies (Supplementary Figure S8), most likely because there were no granulocytes present.
In contrast to the observed cytokine induction in isolated PBMCs, in whole porcine blood infected with S. suis 10ΔdltA for 2h, we found IL-1β but not TNF-α levels to be reduced compared to blood infected with the wt (Figure 6; Supplementary Figure S4). This finding suggests a possible influence of LTA d-alanylation on inflammasome regulation, as TNF-α production and secretion are not inflammasome-dependent. Interestingly, direct recognition of cytosolic LTA of Listeria monocytogenes by the NLRP6 inflammasome was described for macrophages of mice (Hara et al., 2018), while the impact of d-alanylation was not investigated in this context. Since the reduction in IL-1β levels was only visible in whole blood, but not in isolated PBMCs, we speculate that a crosstalk between different immune cells in porcine blood might be important for this phenotype.

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

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

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AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

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