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Vaccination with the immunoglobulin M-degrading enzyme of *Streptococcus suis*, Ide_{Ssuis}, leads to protection against a highly virulent serotype 9 strain

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ABSTRACT

Vaccination of weaning piglets with the recombinant IgM degrading enzyme of *Streptococcus suis* (*S. suis*), $rIde_{Ssuis}$, elicits protection against disease caused by serotype (*cps*) *2* infection. In Europe, *S. suis cps9* is at least as important as *cps2* in causing severe herd problems associated with meningitis, septicemia and arthritis. The objective of this study was to determine humoral and cellular immunogenicities of $rIde_{Ssuis}$ suckling piglet vaccination and to investigate protection against a virulent *cps9* strain. Vaccination in the 2nd and 4th week of life with $rIde_{Ssuis}$ and an oil-in-water adjuvant induced seroconversion against Ide_{Ssuis} in 13 of 20 vaccinated piglets. In the 5th week, survival of the *S. suis cps9* strain was significantly reduced in the blood of prime-booster vaccinated piglets. After a 2nd booster vaccination Ide_{Ssuis}-reactive T helper (Th) cells partially producing TNF-α, IL-17A or IFN-γ were detectable in $rIde_{Ssuis}$-vaccinated but not in placebo-treated piglets and frequencies of Ide_{Ssuis}-reactive Th cells correlated with α-Ide_{Ssuis}–IgG levels. An intravenous challenge, conducted with a *cps9* strain of sequence type (ST) 94, led to 89% mortality in placebo-treated piglets due to septicemia and meningitis. In contrast, all $rIde_{Ssuis}$ prime-booster-booster vaccinated littermates survived the challenge despite signs of disease such as fever and lameness. In conclusion, the described $rIde_{Ssuis}$ vaccination induces humoral and detectable Ide_{Ssuis}-reactive Th cell responses and leads to protection against a highly virulent *cps9* strain.

1. Introduction

S. suis cps9 has become the most important serotype in some European countries with a huge pig industry such as Spain and the Netherlands [1,2]. In the field, prophylaxis against *cps9* is very problematic, because vaccination with a bacterin protects against mortality, but not morbidity [3] and does not reduce colonization and transmission [4]. Furthermore, *cps9* is a strong biofilm inducer and endocarditis is a main manifestation, which might also occur in vaccinated piglets [3,5,6].

A few *S. suis* proteins have been shown to provide protection against *cps2* challenge in pigs, including a combination of muramidase-released protein (MRP) and extracellular factor (EF) [7], surface antigen one (SAO) [8], HP0197 [9], SsPepO [10] and Ide_{Ssuis} [11]. Interestingly, only SAO has been shown to protect against a further serotype, namely *cps1* [12]. However, most *cps1* strains are closely related to *cps2*. Strains of both serotypes often belong to clonal complex (CC) 1 [13]. Noteworthy, other independent studies have shown that piglets with high antibody titers against SAO are highly susceptible to *S. suis* challenge and that these antibodies are not opsonizing [14,15]. Thus, it is currently unknown whether any of the identified protective antigens has the cross-protective potential needed for a universal *S. suis* vaccine.

Different *S. suis* serotypes express a highly specific immunoglobulin M-degrading enzyme, designated Ide_{Ssuis} [16]. The protein is homologous to the IgG protease IdeS of *S. pyogenes* [17], but cleaves solely class M antibodies of swine. Mutants expressing no or a point-mutated Ide_{Ssuis} variant deficient in IgM cleavage show enhanced deposition of C3b on the bacterial surface indicating that IgM cleavage by Ide_{Ssuis} is involved in complement evasion [18,19]. Vaccination of weaning piglets with $rIde_{Ssuis}$ elicits

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antibodies neutralizing the IgM protease activity and protects against morbidity and mortality induced by *cps2* challenge [11].

Read out parameters in vaccination trials with piglets focused on humoral immunity. However, cellular immunity is known to be crucial to restrict colonization of the respiratory tract by the related pathogen *S. pneumoniae* [20,21]. Mice lacking CD4 were found to be more susceptible to systemic *S. suis* infection at a low infection dose [22], whereas in pigs the T cell immune response is not well characterized.

Here, we investigated humoral and cellular immunogenicities of Ide_{Ssuis} vaccinated suckling piglets, being the age class of choice for vaccination in the field. Furthermore, we asked if immunization with Ide_{Ssuis} protects against *S. suis cps9*, the most important and troublesome pathotype in Europe.

2. Materials and methods

2.1. Bacterial strains, growth conditions and profiling of virulence-associated factors

S. suis strain A3286/94 is a *mrp** *sly*$^+$ *cps9* strain belonging to ST99 of CC16 originally isolated from a pig with meningitis [3,15,23,24]. Strains 15-3/3, V5404/2 and 16085/3b were originally isolated from inner organs of diseased piglets with meningitis and/or septicemia and identified as *mrp*$^+$ *sly*$^+$ *cps9*$^+$ in a described multiplex (MP) PCR [25]. *S. suis* strain 10 (*mrp*$^+$ *epf*$^+$ *sly*$^+$) is a virulent serotype 2 strain [11,15,26]. The isogenic mutant 10Δ*ide*$_{Ssuis}$ deficient in IgM cleavage was included in the bactericidal assay to reveal effects mediated by antigen-specific immunity [11,16]. *S. suis* was grown on Columbia agar plates supplemented with 6% sheep blood or in Bacto™ Todd Hewitt broth (THB). *Escherichia coli* (*E. coli*) strains were cultured in Luria-Bertani (LB) medium including 100 μg/ml ampicillin, if appropriate.

2.2. Expression and purification of recombinant (r) proteins

The expression and the purification of His-tagged $rIde_{Ssuis}$ of serotype 2 strain 10 [16] and His-tagged fibronectin binding domain of streptococcal fibronectin-binding protein I of *Streptococcus pyogenes* (SfbI) [27] was performed as described previously [16]. SfbI was chosen as a control protein as *Streptococcus pyogenes* is not found in pigs.

2.3. Animal experiments

Piglets were infected experimentally and cared for in accordance with the principles outlined in the EU Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). All animal experiments or samplings were conducted by veterinarians and in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes and the German Animal Protection Law (Tierschutzgesetz). The animal experiment of this study was approved by the Landesdirektion Sachsen (permit no. TVV28/16), which includes approval through the registered committee for animal experiments. The collection of blood samples was approved by the Landesdirektion Sachsen (permit no. N01/16 and N19/14).

Five weeks prior to farrowing four pregnant sows of a German Landrace pig herd considered to be free of *cps9*$^+$ *S. suis* strains were transported to the Faculty of Veterinary Medicine, Leipzig University. The classification as *cps9* free was based on the genotyping results including a described MP-PCR [25] of *S. suis* isolates from the tonsils of more than 400 animals over the last 14 years. After birth the suckling piglets were treated as follows: Piglets of one litter were equally distributed to the vaccination and the placebo group with a final number of 20 piglets per group. At an age of 2 weeks a vaccine containing $rIde_{Ssuis}$ as antigen or a placebo was applied intramuscularly, both supplemented with 20% [vol/vol] Emulsigen as adjuvant. The piglets were boostered 14 days later. Nine piglets per group were boostered a second time another 14 days later. One dose of $rIde_{Ssuis}$ vaccination contained 0.4 mg $rIde_{Ssuis}$. After the first booster vaccination at an age of 4 weeks, piglets were weaned and moved to the trial pen. All piglets (n = 18) used for experimental infection were placed in one pen. The person (CGB) who made the final decision of euthanasia did not know whether a specific piglet was vaccinated with $rIde_{Ssuis}$ or placebo-treated (partially blinded experiment). Piglets were challenged intravenously at an age of 8 to 9 weeks (17 days after the second booster) with 2×10^8 CFU of *S. suis* strain 16085/3b grown in Bacto™ Tryptic Soy Broth (TSB) without dextrose. Post infection, the health status of the animals was monitored every 8 h, including measurement of the inner body temperature, assessment of movements and feed intake (piglets were only fed at these time points). Based on predefined criteria (Table S1) clinical signs were scored. Piglets were classified as morbid if a body temperature of ≥40.2 °C or/and severe clinical signs of an acute disease were observed. In case of high fever (≥40.5 °C), apathy and anorexia persisting over 32 h as well as in all cases of central nervous system dysfunction or clinical signs of acute polyarthritis, animals were euthanized for animal welfare reasons. All surviving piglets were sacrificed 14 days post infection (dpi). After euthanasia every animal went through the same procedure of necropsy to collect the following samples for histological (h) and semi-quantitative bacteriological (b) investigations as described previously [3,15]: cerebrospinal fluid (b); brain (b, h); tarsal and carpal joints (b, h), peritoneal, pleural and pericardial swabs (b), peritoneum, pleura and pericardium (h); cranial lobe of the left lung (b, h); liver (b, h); spleen (b, h); mitral valve (b, h) and tonsil (b, h). The histological screenings were scored as described [28] and briefly mentioned in the footnotes of Table 2. Isolation of the challenge strain was confirmed by MP-PCR detecting *mrp*, *epf*, *sly*, *arc*A, *gdh*, *cps1*, *cps2*, *cps7* and *cps9* [25].

2.4. Bactericidal assay

Survival of *S. suis* in porcine blood *ex vivo* was determined as previously described [11]. Briefly, 500 μl of heparinized blood (16 I. U. heparin/ml) was mixed with 6×10^5 CFU of exponentially grown bacteria (OD_{600}: 0.5–0.6). The samples were incubated for 2 h at 37°C on a rotator. Blood for bactericidal assays was drawn from all piglets of the vaccination trial 7 and 17 days after first and second booster vaccinations, respectively. The bactericidal assays were conducted within 4 h after blood collection. The specific bacterial contents in CFU/ml were determined by plating serial dilutions at t = 0 min and t = 120 min and the survival factor of *S. suis* for each sample was calculated by dividing the two values.

2.5. Detection of anti (α) -Ide_{Ssuis} IgG

The detection of α-Ide_{Ssuis} IgG was performed as previously described [11]. Sera of piglets immunized with $rIde_{Ssuis}$ and a truncated derivative ($rIde_{Ssuis}$–homologue) in the previous study served as reference serum and positive control in the α-Ide_{Ssuis} ELISA, respectively.

2.6. Detection of Ide_{Ssuis}-reactive Th-cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation on Biocoll (Merck-Biochrom, Berlin, Germany) and cryo-conserved at

−80 °C until usage for restimulation. PBMCs were cultivated in 96-well plates (1×10^6 cells/well) in complete Iscove's Modified Dulbecco's Medium (IMDM, Pan Biotech, Aidenbach, Germany) containing 10% FCS (Gibco) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively; Merck-Biochrome, Berlin, Germany). Antigen-specific restimulation was conducted with 5 μg/ml $rIde_{Ssuis}$ or 5 μg/ml His-tagged rSfbI [27] as a non-relevant control antigen. Both recombinant antigens were expressed in *E. coli* and purified via Ni-affinity chromatography. rSfbI was included as a control to estimate background reactivity. PMA/Ionomycin (10 ng/ml and 500 ng/ml, respectively, both purchased from Merck-SIGMA-Aldrich, Taufkirchen, Germany) were used as a positive control added for the last 4 h of incubation time and medium alone was used as a negative control. PBMCs were stimulated for 18 h in presence of 2 μg/ml Brefeldin A (Enzo Life Science, Lörrach, Germany) for the last 14 h at 37°C, 5% CO_2 in a humidified atmosphere. To label cells for flow cytometry analysis, the stimulated cells were harvested and stained as described in the section flow cytometry and as previously shown [29]. Measurement of samples was conducted at LSR-Fortessa™ (Beckton Dickinson, Germany, Heidelberg) recording $3–5 \times 10^5$ viable $CD3^+CD4^+$ cells. The frequency of antigen-reactive Th cells ($CD154^+$) was determined from the antigen-experienced Th cell population, described as $CD3^+CD4^+CD8\alpha^+$ cells. Frequency of Ide_{Ssuis}-induced Th cells was calculated as the difference of the percentage of $CD154^+$ cells after Ide_{Ssuis} re-stimulation minus the percentage of $CD154^+$ cells of medium cultivated cells from the same sample.

2.7. Flow cytometry staining

To label antigen-reactive Th cells for flow cytometry analysis $6x10^6$ PBMCs per stimuli were used after antigen-specific restimulation. Cells were washed two times with PBS before addition of fixable viability dye eFlour™ 506 (1:500, Thermo Fisher Scientific, San Diego, CA). For extracellular staining anti-porcine CD3-PE ~ Cy7 (clone: BB23-8E6-8C8), anti-porcine CD4-PE (clone: 74–12-4) and anti-porcine CD8α-FITC (clone: 76-2-11), all purchased from Beckton Dickinson (BD, Heidelberg, Germany), were used. Following fixation (2% paraformaldehyde) the cells were permeabilized in PBS buffer containing 3% FCS, 0.1% NaN3 and 0.5% saponin for intracellular staining with anti-human CD154-VioBlue (clone: 5c8, Miltenyi, Colone, Germany), anti-porcine IFN-γ-PerCP ~ Cy5.5 (clone: P2G10, BD), anti-human IL-17A-AlexaFlour®647 (clone: SCPL1362, BD) and anti-human TNF-α-BV421 (clone: MAb11, BioLegend, San Diego, CA).

2.8. Statistical analysis

The evaluation of more than two groups was carried out using one- or two- way analysis of variance (ANOVA) or Kruskall-Wallis with a subsequent Tukey's or Dunn's multiple comparisons test, respectively. Differences between two groups were analyzed with the Mann-Whitney *U* test. Correlation was calculated with the Pearson test. The Wilcoxon test was used for comparison of different time point values within the same group in case of no more than two repeated measures. The data presented in the Kaplan-Meier-diagrams were analyzed with the log rank test. Means and standard deviations of the results are shown. All statistical tests were conducted with GraphPad Prism 7.01 software. Probabilities lower than 0.05 were considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

3. Results

3.1. S. suis 16085/3b exhibits increased bacterial survival in porcine blood in comparison to other cps9 strains

In Europe, *S. suis cps9* is a major porcine pathogen causing herd problems associated mainly with meningitis, endocarditis and arthritis [2,25]. Recently, a herd experienced an escalating *S. suis cps9* problem in growing piglets due to meningitis and septicemia associated with cyanosis and sudden death. As the extent and severity of disease on this farm appeared extreme, the *S. suis cps9* strain 16085/3b isolated from the spleen of a pig from this farm was compared to other *S. suis cps9* strains by profiling of virulence-associated factors, multi locus sequence typing (MLST) and analysis of bacterial survival in porcine blood. Interestingly, strain 16085/3b is a mrp^+ sly^+ $cps9^+$ strain of ST94 and thus genetically distinct to other invasive *cps9* strains. As shown in Fig. 1 *S. suis cps9* strain 16085/3b shows significantly higher bacterial survival factors than the other three tested *cps9* strains of ST16 (V5402/2 and 15-3/3) and ST99 (A3286/94) in blood from *cps9* free piglets at an age of 6.5, 7.5 and 8.5 weeks. In addition, for the 16085/3b strain we observed a significant increase of bacterial

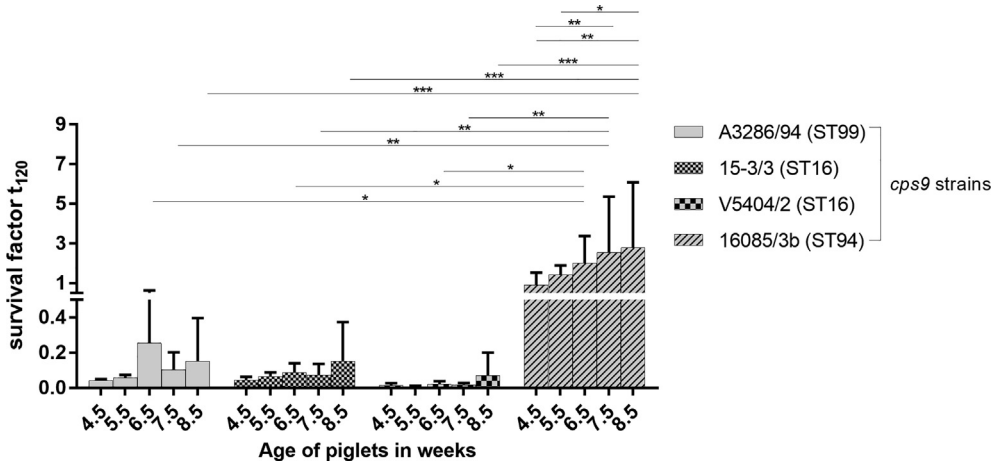


Fig. 1. Strain 16085/3b shows increased survival in porcine blood compared to other *cps9* strains. Bactericidal assays were conducted with *S. suis cps9* strains A3286/94, 15-3/3, V5404/2 and 16085/3b belonging to the indicated sequence types (ST) with blood drawn from the same *S. suis cps9* free piglets (n = 5) at the specified ages. Note, that data of strain 16085/3b was published recently [36]. The survival factor represents the ratio of CFU at 120 min to CFU at time zero. Bars and error bars represent mean values and standard deviations, respectively. Significant differences were determined using two-way ANOVA and a subsequent Tukey's multiple comparisons test. Significances are indicated ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

survival in blood of 8.5 week-old (mean = 2.8, SD = 3.3, n = 5) piglets in comparison to 4.5 week-old (mean = 0.9, SD = 0.6, n = 5) and 5.5 week-old piglets (mean = 1.4, SD = 0.5, n = 5) (Fig. 1). Due to this phenotype, we chose strain 16085/3b as challenge strain for this vaccination study, since previous experimental infection studies with other *cps9* strains led to rather low rates of mortality making it difficult or even impossible to draw conclusions on protection against mortality [3,4].

3.2. Early prime-booster vaccination of piglets in the 2nd and 4th week with rIde$_{Ssuis}$ elicits specific IgG antibody titers and bactericidal immunity against S. suis cps9 strain 16085/3b

Induction of early protective immunity against *S. suis* by suckling piglet vaccination would be advantageous over weaning piglet vaccination, as diseases might occur after weaning. Thus, we investigated humoral immunogenicities of early prime-booster rIde$_{Ssuis}$ vaccination in the 2nd and 4th week of life, respectively. Prior to vaccination in the 2nd week of life, all suckling piglets had α-Ide$_{Ssuis}$ IgG levels below 5 ELISA units (mean = 1.7; SD = 0.9; n = 20, Fig. 2). Seven days after the 1st booster vaccination, at an age of 5 weeks, a mean of 20.9 ELISA units (SD = 14.4, n = 20) α-Ide$_{Ssuis}$ IgG was recorded in vaccinated piglets, which is significantly higher than the antibody level found in their placebo-treated littermates (mean = 0.5 ELISA units, SD = 0.2, n = 20; Fig. 2). However, 7 of 20 vaccinated piglets showed no or only a low increase in α-Ide$_{Ssuis}$ IgGs with a difference of less than 10 ELISA units between the pre and post vaccination-serum.

A bactericidal assay conducted 7 days after first booster vaccination revealed significantly lower survival factors of the *cps9* strain 16085/3b in the blood of the vaccinated piglets (mean = 0.3, SD = 0.2, n = 20) compared to the blood of placebo-treated piglets (mean = 0.5, SD = 0.3, n = 20; Fig. 3). The *cps2* wt strain 10 also had a lower survival factor in the blood of vaccinated piglets (mean = 0.2, SD = 0.3, n = 20) in comparison to placebo-treated piglets (mean = 0.3, SD = 0.5, n = 20), though differences were not significant (Fig. 3). In conclusion, an early prime-booster Ide$_{Ssuis}$ vaccination (in the 2nd and 4th week of life) leads to an increase of α-Ide$_{Ssuis}$-IgG in serum at an age of 5 weeks which is associated with reduced survival of *S. suis* in blood.

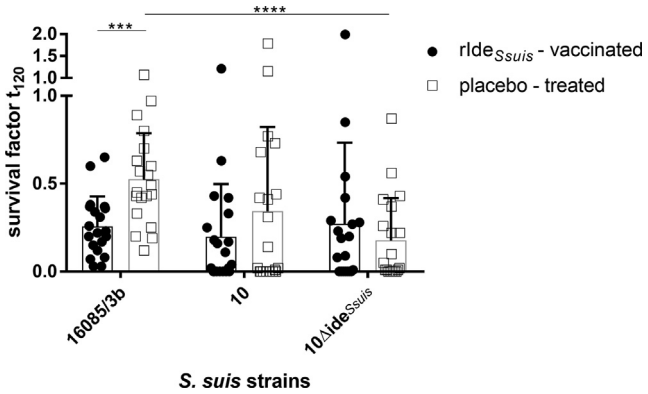


Fig. 3. Survival of *S. suis cps9* strain 16085/3b is significantly reduced in blood of rIde$_{Ssuis}$-vaccinated piglets (n = 20) in comparison to placebo-treated littermates 7 days after the first booster vaccination. Bacterial survival of *cps2* strain 10 and its isogenic mutant 10Δ*ide*$_{Ssuis}$ was also determined in the blood of the same piglets. Bars and error bars represent mean values and standard deviations, respectively. The Mann-Whitney-*U* test was used for comparison of rIde$_{Ssuis}$-vaccinated versus placebo-treated group. Two-way ANOVA and Tukey's multiple comparisons post-hoc test were used for comparison of survival factors of the three *S. suis* strains per group. Significant differences are indicated (***$p < 0.001$, ****$p < 0.0001$).

3.3. Vaccination with rIde$_{Ssuis}$ protects growing piglets against mortality caused by S. suis cps9 infection

In contrast to the results of a previous vaccination trial with weaning piglets [11], early prime-booster vaccination with rIde$_{Ssuis}$ did not elicit a prominent systemic IgG response against rIde$_{Ssuis}$ in all vaccinated piglets. Piglets with a prominent IgG response against rIde$_{Ssuis}$ following prime-booster vaccination, which additionally showed neutralization of the IgM protease in Western blot analysis (as described previously by Seele et al., 2015), were chosen for another experiment not dealing with *cps9* and thus not part

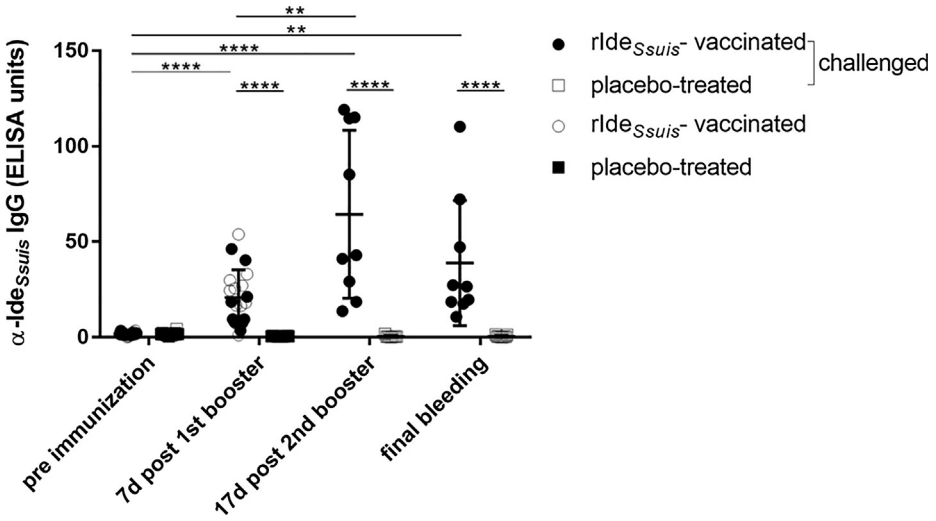


Fig. 2. rIde$_{Ssuis}$ vaccination induces specific IgG antibodies in serum. Time course of α-Ide$_{Ssuis}$ IgG antibodies in rIde$_{Ssuis}$-vaccinated and placebo-treated piglets during prime-booster-booster vaccination and after *S. suis cps9* infection. IgG levels were determined in the 2nd week of life (pre immunization) and 7 days after the first booster in rIde$_{Ssuis}$-vaccinated (○, ●) and placebo-treated (■, □) piglets (n = 20/group). In the 6th week of life, nine piglets per group were boostered (●) or placebo-treated (□), respectively, for a second time and challenged 17 days later. Blood samples taken from euthanized piglets are indicated as final bleeding and include the time period from 1 until 14 dpi. Mean values are indicated by horizontal lines, standard deviations by error bars. Statistical analyses were conducted with the Mann-Whitney *U* test (placebo-treated vs. rIde$_{Ssuis}$-vaccinated), the Wilcoxon test (comparison of pre immunization and 7d post 1st booster immune sera, n = 20/group) or the one-way ANOVA and subsequently the Dunn's multiple comparisons test (pre and post immune sera of the 18 piglets challenged with *S. suis cps9*). Significant differences are indicated (**$p < 0.01$, ****$p < 0.0001$).

of the study described here. A 2nd booster was applied to the remaining nine prime-booster vaccinated piglets which apart from two animals (>40 α-Ide$_{Ssuis}$ IgG ELISA units) had responded poorly (<22 α-Ide$_{Ssuis}$ IgG ELISA units) to the vaccine in terms of seroconversion against rIde$_{Ssuis}$ (Table S2). The 2nd booster led to a significant increase of the α-Ide$_{Ssuis}$ IgG level to a mean of 64.4 ELISA units (SD = 43.9, n = 9) 17 days after the 2nd booster in the 8th week of life compared to a mean of 18.2 ELISA units (SD = 15.3, n = 9) 7 days after the 1st booster in these nine piglets (Fig. 2). These prime-booster-booster vaccinated piglets (n = 9) were used for the challenge experiment. A bactericidal assay conducted after the 2nd booster immunization suggested killing of the *cps9* strain with a mean survival factor of 0.6 (SD = 0.6) in the rIde$_{Ssuis}$-immunized group in contrast to substantially higher survival in placebo-treated littermates with a mean survival factor of 1.3 (SD = 1.15), respectively, although these differences were statistically not significant (Fig. S1). The intravenous challenge with the *cps9* strain 16085/3b was conducted on the same day to test protection against this important serotype. A clinical scoring system was applied as shown in Table S1. Six of nine placebo-treated piglets died or were killed after reaching a clinical score of 25 within 32 h after experimental infection (Fig. 4). Four of them displayed a peracute course of disease with the inability to rise, pain vocalization, vomiting and apathy within less than 8 h after challenge. Further two showed signs of central nervous system dysfunction including convulsions, opisthotonus and tremor the day after challenge (Table 1). Another placebo-treated piglet showed signs of central nervous system dysfunction as late as 14 dpi. In contrast, none of the nine vaccinated piglets died or showed signs of acute septicemia, central nervous system dysfunction or the inability to rise in the observation period after the *cps9* challenge. Only one vaccinated piglet received a clinical score above 10 after experimental infection. However, short time fever during the first three days following the challenge were recorded in seven of nine vaccinated piglets (Fig. S2) and lameness was observed in four of nine piglets. These piglets recovered within 24–32 h in all cases except for one. Thus, prime-booster-booster rIde$_{Ssuis}$ vaccination protects piglets against mortality but not morbidity in this intravenous *cps9* challenge model.

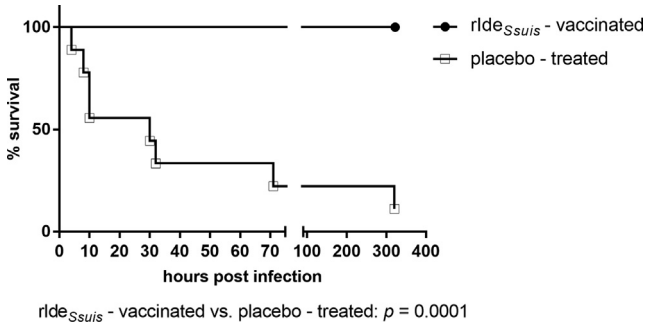


Fig. 4. rIde$_{Ssuis}$ vaccination protects against mortality caused by *S. suis cps9* challenge. The Kaplan-Meier diagram shows mortality of rIde$_{Ssuis}$ prime-booster-booster (n = 9) and placebo-treated (n = 9) growing piglets after infection. Piglets were challenged through intravenous application of 2×10^8 CFU of *S. suis* strain 16085/3b (*mrp** *sly*$^+$ *cps9*) 17 days after 2nd booster immunization. In case of high fever (≥40.5 °C), apathy and anorexia persisting over 32 h as well as in all cases of central nervous system dysfunction or clinical signs of acute polyarthritis, animals were euthanized for animal welfare reasons. All surviving piglets were sacrificed 14 days post infection (dpi). Statistical analysis was conducted with the log-rank test (p value is shown below the diagram).

The histological screening revealed that six of nine placebo-treated piglets had typical fibrinosuppurative lesions in at least two inner organs (Table 2). Accordingly, *S. suis cps9* was detected in seven of nine placebo-treated piglets in two or more inner organs indicating severe bacteremia or infection of multiple organs after challenge (Table 3). The pathological score ω was substantially lower in rIde$_{Ssuis}$-vaccinated piglets (2.0 versus 3.0) as moderate and severe fibrinosuppurative lesions were not detected in the brain of these animals (Table 2). Furthermore, the challenge strain was isolated from an inner organ of only one rIde$_{Ssuis}$-vaccinated piglet (Table 3). In conclusion, recording of fibrinosuppurative inflammations and bacteriology indicated protection in prime-booster-booster rIde$_{Ssuis}$-vaccinated piglets against meningitis and bacterial dissemination.

3.4. Prime-booster-booster rIde$_{Ssuis}$ vaccination elicits a detectable antigen-reactive Th cell response

We recently described a method to detect antigen-reactive Th cell responses in pigs using the transiently expressed Th cell activation marker CD154 [29]. Therefore we analysed antigen-experienced Th cells (pre-gated for lymphocytes > single cells > viable CD3$^+$ > CD4$^+$ CD8α$^+$), described as CD3$^+$CD4$^+$CD8α$^+$ [30,31], shown in Fig. 5A. In comparison to piglets of the placebo-group the re-stimulation of PBMCs of rIde$_{Ssuis}$-immunized piglets with rIde$_{Ssuis}$ induced a significantly increased number of CD154$^+$ Th cells (Fig. 5B, upper left) within the antigen-experienced Th cell population. In contrast, stimulation with a non-relevant control antigen (recombinant fibronectin binding domain of SfbI) did not induce CD154$^+$ Th cells in both groups that confirm the antigen-specific reactivity of antigen-experienced Th cells in the assay. Considering the cytokine profile of the rIde$_{Ssuis}$-reactive CD154$^+$ Th cells, we found a significantly increased number of TNF-α (Fig. 5B, upper right) and IL-17A producer (Fig. 5B, lower left) in the vaccination group in comparison to placebo-treated animals. The same trend was found for IFN-γ producing CD154$^+$ Th cells, but not statistically significant (Fig. 5B, lower right).

To analyze, whether the detected Ide$_{Ssuis}$-reactive Th cell frequencies are linked to the B cell-mediated Ide$_{Ssuis}$-specific IgG response described above (Fig. 2), correlation of the frequencies of rIde$_{Ssuis}$-induced antigen-reactive CD154$^+$ Th cell frequencies (representing the difference of Ide$_{Ssuis}$-stimulation minus medium control of the same sample) and Ide$_{Ssuis}$-specific IgG levels was performed using the Pearson test. In contrast to the placebo group

Table 1
Assessment of the protection induced by the indicated treatments against morbidity and mortality after intravenous *S. suis* serotype 9 challenge.

Immunization antigen	Morbidity	Mortality	Mean clinical score[e] (SD)	Clinical signs			Max. body temperature (°C)		
				CNS[a]	Lameness	no feed intakee	<40	≥40 and ≤40.2	>40.2
placebo	8/9	8[b]/9	21 (8.1)	4/9	3[c]/9	4(6)[d]/9	1[d]/9	0[d]/9	6[d]/9
rIde$_{Ssuis}$	8/9	0/9	6 (3.9)	0/9	4/9	1/9	1/9	1/9	7/9

[a] Signs of central nervous system (CNS) dysfunction such as convulsions and opisthotonus.
[b] One placebo-treated piglet showed central nervous system dysfunction and reached a score of 25 on the last day of the experiment.
[c] Three additional piglets became recumbent.
[d] Two piglets died a few hours after experimental infection prior to the first feeding and measurement of body temperature.
[e] For the detailed clinical scoring system see Table S1.

Table 2
Scoring of fibrinosuppurative lesions of piglets challenged with *S. suis cps*9 strain 16085/3b.

Immunization antigen	Piglets without lesions[a]	Piglets with lesions in two or more locations[a]	Brain meningitis, chorioiditis			Serosae pleuritis or peritonitis or pericarditis			Joint synovialitis			Spleen and liver splenitis[b] or hepatitis			Lung pneumonia			Heart endocarditis			
			5[c]	3[d]	1[e]	4[c]	2[d]	1[e]	4[c]	2[d]	1[e]	4[c]	2[d]	1[e]	4[c]	2[d]	1[e]	4[c]	2[d]	1[e]	ω[f]
placebo	0/9	6/9	3/9	1/9[g]	0/9	0/9	3/9	1/9	1/9	2/9	0/9	0/9	6/9	1/9	0/9	0/9	1/9	0/9	3/9	1/9	**3.0**[h]
rIde$_{Ssuis}$	3/9	2/9	0/9	0/9	2/9	0/9	1/9	0/9	1/9	3/9	0/9	0/9	0/9	6/9	0/9	0/9	0/9	1/9	2/9	2/9	**2.0**

[a] Only fibrinosuppurative lesions are considered. Individual single perivascular neutrophils are not counted.
[b] Neutrophilic accumulation of the splenic red pulp.
[c] Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative inflammations.
[d] Scoring of 2 and 3 indicates mild focal fibrinosuppurative inflammation.
[e] Individual single perivascular neutrophils received a score of 1.
[f] $\omega = \Sigma score_{max}/n_{animals}$.
[g] diffuse, mild plexus choroiditis.
[h] Four of the nine placebo-treated piglets died within 8 h after experimental infection. These piglets reached comparable low histological scores of 2 (n = 3) and 3 (n = 1).

Table 3
Reisolation of the challenge strain from piglets after intravenous challenge with *S. suis cps9* strain 16085/3b.

Immunization antigen	Number of piglets positive for the isolation of the challenge strain in an inner organ[b] or in serosa or in joint fluid	Number of piglets positive for the isolation of the challenge strain in ≥ 3 inner organs[b]	Number of piglets in which the *S.suis* challenge strain[a] was isolated from							
			Tonsils	Lung[c]	Serosa[d]	Spleen	Liver	Brain, CSF[e]	Joint fluid[f]	Endocard
placebo	8/9	7/9	0/9	5/9	4/9	7/9	5/9	7/9	6/9	6/9
rIde$_{Ssuis}$	1/9	0/9	0/9	0/9	0/9	0/9	1/9	0/9	0/9	1/9

[a] The challenge strain was identified by PCR.
[b] Inner organ refers to lung, spleen, liver, brain, CSF or endocard but not the tonsils.
[c] One cranial lobe was investigated.
[d] Pleural, peritoneal or pericardial cavity.
[e] Cerebrospinal fluid.
[f] Punctures of both tarsal and carpal joints were investigated in each animal. In case of lameness additional joint punctures of the respective limb were screened.

($r^2 = 0.10$), within the rIde$_{Ssuis}$-vaccinated group (n = 9) the number of Ide$_{Ssuis}$-reactive CD154$^+$ Th cells correlated with the Ide$_{Ssuis}$-specific IgG level ($r^2 = 0.48$) in this manner that animals with higher frequencies of rIde$_{Ssuis}$-induced CD154$^+$ Th cells showed higher levels of anti-Ide$_{Ssuis}$-IgGs (Fig. 5B). Taken together, rIde$_{Ssuis}$-prime-booster-booster vaccination of piglets induces a Th cell response that is linked to Ide$_{Ssuis}$-specific IgG level.

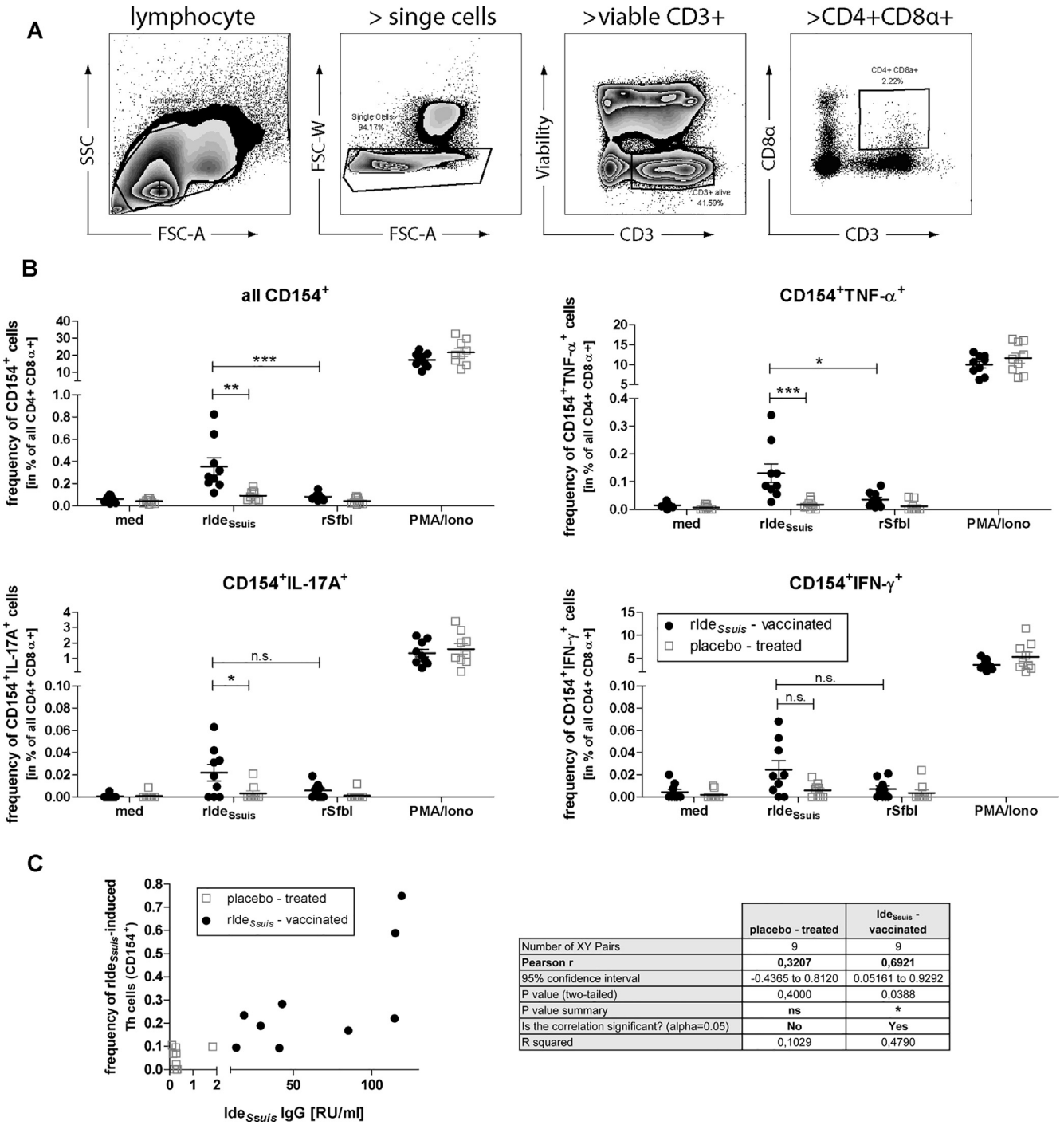
In addition, we analyzed the frequency of Ide$_{Ssuis}$-reactive Th cells after challenge to prove whether vaccination-induced Th cells are re-activated by the challenge with *cps9* strain 16085/3b. Therefore the two placebo-treated animals that survived until day 14 and five rIde$_{Ssuis}$-immunized piglets, all with a maximal cumulative clinical score ≥ 4 were selected. We could not detect an increase of Ide$_{Ssuis}$-reactive Th cells after challenge, neither for the frequency of all rIde$_{Ssuis}$-induced CD154$^+$ cells nor for a cytokine producing subtype of them (Fig. S3A). Rather, by trend we observed a reduced number of Ide$_{Ssuis}$-reactive Th. We found a similar result for the α-Ide$_{Ssuis}$ IgG levels, which did not increase after the challenge, but decreased in tendency (Fig. S3B).

In summary we observed an induction of Ide$_{Ssuis}$-reactive Th cells by immunization, but no recall upon challenge infection.

4. Discussion

S. suis cps2 and *9* have an enormous impact on animal health in Europe. A vaccine protecting against both serotypes is needed in the field to ensure return of investments in the pig industry and improve animal health and welfare. Though *cps9* is a major porcine pathogen in the field, no recombinant *S. suis* vaccine has been tested in a challenge experiment with a *cps9* strain [32]. This study revealed protection through prime-booster-booster vaccination with rIde$_{Ssuis}$ against mortality induced by intravenous application of a *S. suis cps9* strain. rIde$_{Ssuis}$ vaccination has been shown to protect piglets also against *S. suis cps2* challenge [11]. Thus, Ide$_{Ssuis}$ is a cross-protective antigen covering at least related *cps2* and *cps9* strains. In contrast to the previous study including an intranasal *cps2* challenge, we did not observe protection against morbidity following intravenous *cps9* challenge, as vaccinated piglets showed temporarily elevated body temperature, reduced feed intake and lameness. However, sudden death, recumbency, convulsions and polyarthritis were observed in placebo-treated piglets only. As these signs occurred a few hours after challenge in contrast to the intranasal *cps2* challenge, the intravenous application of the *cps9* strain 16085/3b with 2×10^8 CFU conducted in this study is regarded as a very hard challenge. Noteworthy, sudden death is not a common sign in experimental infections with *S. suis*. Based on the rapid progression of disease and the high rate of mortality after challenge as well as the increased survival of this *cps9* strain in porcine blood in comparison to the other investigated *cps9* strains, strain 16085/3b is considered very virulent. MLST revealed that this strain is a ST94 strain which in contrast to other *cps9* strains such as A3286/94 (ST99), V5402/2 and 15-3/3 (both ST16) does not belong to CC16. We cannot rule out differences in protective efficacies of rIde$_{Ssuis}$ vaccination against *cps9* strains of different clonal complexes. However, experimental infections with *cps9* strains of CC16 have resulted in low morbidity, making it very difficult if not impossible to draw conclusions on protection [4].

Pathohistological screenings revealed fibrinosuppurative meningitis only in placebo-treated piglets (4/9) and in accordance a substantially lower pathohistological score in rIde$_{Ssuis}$-vaccinated piglets (ω = 2.0 versus ω = 3.0, respectively). Of note, meningitis is the most important pathology of *S. suis* infection in weaning piglets causing high numbers of losses [33]. Though fibrinous endocarditis



	placebo - treated	Ide Ssuis - vaccinated
Number of XY Pairs	9	9
Pearson r	**0,3207**	**0,6921**
95% confidence interval	-0.4365 to 0.8120	0.05161 to 0.9292
P value (two-tailed)	0,4000	0,0388
P value summary	**ns**	*****
Is the correlation significant? (alpha=0.05)	**No**	**Yes**
R squared	0,1029	0,4790

Fig. 5. Vaccination-induced Ide$_{Ssuis}$-reactive Th cell frequencies correlate with Ide$_{Ssuis}$-specific IgG levels. (A) Gating strategy to pre-gate antigen experienced Th cells (CD3+CD4+CD8α+). (B) Within this population frequency of Ide$_{Ssuis}$-reactive Th cells was determined 17 d post 2nd booster immunization (prior *cps9* challenge). Therefore PBMCs from rIde$_{Ssuis}$- vaccinated and placebo-treated pigs (n = 9 per group) were stimulated with 5 μg/ml rIde$_{Ssuis}$ or the recombinant fibronectin binding domain of SfbI as a non-relevant control antigen for 18 h and in presence of Brefeldin A (2 μg/ml) for the last 14 h to detect intracellular CD154 and cytokine expression (frequency of viable CD3+ cells; mean 41.8 ± SD 5.0%). As controls PMA/ionomycin (10 ng/ml and 500 ng/ml, respectively, positive control) and medium (med, negative control) were used. For statistical analysis non-parametric Kruskal-Wallis test and Dunn's multiple comparisons post-hoc test was used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (C) Pearson correlation between frequencies of Ide$_{Ssuis}$-induced CD154+ Th cells (calculation see materials/methods) and Ide$_{Ssuis}$ IgG levels.

was observed in three placebo-treated and also in three rIde$_{Ssuis}$-vaccinated piglets, the challenge strain was detected on the respective mitral valve in six placebo-treated but only in one rIde$_{Ssuis}$-vaccinated piglet. Whether rIde$_{Ssuis}$ vaccination might be less protective against endocarditis needs to be further clarified. As *S. suis* forms biofilms covered with fibrin on heart valves, killing of streptococci by humoral immunity is very limited once this vegetation is formed [6]. However, as intravenous application bypasses mucosal immunity, rIde$_{Ssuis}$ vaccination might still protect against endocarditis in mucosal infections. Compared to our previous *S. suis cps2* study [11], the pathohistological score in placebo-treated piglets was notably lower (ω = 3.6 versus ω = 3.0, respectively). We suggest that this is due to the sudden death of four piglets on the day of infection. The peracute course of disease

did most likely not leave enough time for severe lesions to develop. The piglets only reached pathohistological scores of 3 at the maximum.

We investigated cellular immunogenicities to find out if antigen-reactive Th cells are elicited by our vaccination protocol. Since Th cell-mediated B cell activation supports the production of highly affine specific IgGs [34], we investigated the correlation of rIde$_{Ssuis}$-reactive Th cell frequencies with rIde$_{Ssuis}$-specific IgG levels. For this Th cell support especially the activity of TfH cells (follicular T-helper cells) in draining lymph nodes is of importance, as recently also demonstrated in pigs [35]. However, lymph node tissue from immunized animals in a vaccination study is usually not accessible. Therefore, it is more beneficial to use readout-parameters for T cell reactivity from blood samples. The results of this study showed that prime-booster-booster vaccination with a vaccine containing rIde$_{Ssuis}$ and 20% Emulsigen results in induction of antigen-reactive Th cells detectable in porcine blood. The antigen-specific reactivity of this read out parameter is confirmed by using a *S. pyogenes* antigen (the fibronectin-binding domain of SfbI) as control that was also expressed as His-tagged protein in *E. coli* and purified via Ni-affinity chromatography [27]. Furthermore, the number of Ide$_{Ssuis}$-reactive CD154^{+} Th cells correlated with the Ide$_{Ssuis}$-specific IgG level within the immunization group. This is in accordance with our previous findings that Ide$_{Ssuis}$-reactive CD154^{+} Th cell frequencies correlated with Ide$_{Ssuis}$-specific IgG levels [29], although in contrast to our previous study, we did not observe a correlation with the frequency of IFN-γ producing CD154^{+} cells. In addition, we analyzed Ide$_{Ssuis}$-reactive Th cells in PBMCs after *cps9* challenge (14 dpi.). Based on our data the adaptive immune response to Ide$_{Ssuis}$ was not recalled by the *S. suis cps9* challenge for both, Ide$_{Ssuis}$-specific-IgGs and Ide$_{Ssuis}$-reactive-Th cells. It is very unlikely that the time point of measurement (14 dpi) was too late after challenge as Ide$_{Ssuis}$-reative Th cells were detectable 17 days post 2nd booster and infection was not restricted to the day of challenge as indicated by the course of body temperature (Fig. S2) and the detection of the challenge strain in the mitral valve of piglet #1 (Table S2). In experiments designed to analyse antigen-specific recall of Th cells by using DO11.10/RagKO mice immunized and infected with Ova-expressing *S. pneumoniae*, Trzciński *et al.* demonstrated that vaccination-induced Th17 cells are only protective against *S. pneumoniae* by antigen-specific recall during challenge infection [21]. However, based on our results we suppose that Ide$_{Ssuis}$ is not an immunodominant T cell antigen under invasive infection and therefore a direct involvement of Ide$_{Ssuis}$-reactive Th cells in protective mechanisms is improbable.

As we could previously demonstrate, the vaccination with rIde$_{Ssuis}$ elicits antibodies neutralizing the IgM protease activity of Ide$_{Ssuis}$ [11]. Recently we demonstrated that the activity of complement is important for clearance of *S. suis* in porcine blood, whereas the complement deposition is reduced by the protease activity of Ide$_{Ssuis}$ [19]. Thus, neutralization of the IgM protease by α-Ide$_{Ssuis}$-IgGs should improve the complement-mediated killing of *S. suis* in porcine blood.

Additionally, in our previous study we found a significantly lower bacterial survival factor of *S. suis* wt in blood of vaccinated piglets in comparison to the respective survival factor of the Ide$_{Ssuis}$ mutant 10Δide$_{Ssuis}$ [11]. In agreement with the location of Ide$_{Ssuis}$ on the bacterial surface [16], the most plausible explanation for this observation is that α-Ide$_{Ssuis}$ antibodies mediate opsonophagocytosis. However, a significant difference was not observed in this study (Figs. 3 and S1).

As mentioned above, prime-booster-booster vaccination was necessary to elicit α-Ide$_{Ssuis}$-IgG levels comparable to those of our previous study. Still, in case of five piglets less than 43 ELISA units were measured at 17 d post 2nd booster (Table S2). α-Ide$_{Ssuis}$-IgG levels and the clinical scores obtained following experimental infection did not show a significant correlation (data not shown). Further studies are necessary to clarify if the data obtained in the described α-Ide$_{Ssuis}$-IgG ELISA allows setting up a threshold for protection against severe disease.

In summary, this study demonstrates protection against *S. suis cps*9 through prime-booster-booster vaccination of piglets with rIde$_{Ssuis}$. This vaccination was associated with induction of α-Ide$_{Ssuis}$-IgG and Ide$_{Ssuis}$-reactive T cells. We suppose that Ide$_{Ssuis}$ -reactive Th cells are important for the B cell immune response to Ide$_{Ssuis}$, but recall of Ide$_{Ssuis}$ -reactive Th cells was not detectable after challenge and is thus not considered to be crucial for protection.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This study was financially supported by IDT Biologika GmbH. IDT Biologika GmbH applied for a European patent concerning an Ide$_{Ssuis}$-based *S. suis* vaccine (Nr. 14 170 637.4).

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Appendix A. Supplementary material

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