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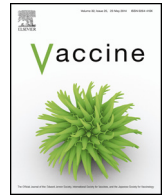
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Vaccination of pigs with attenuated *Lawsonia intracellularis* induced acute phase protein responses and primed cell-mediated immunity without reduction in bacterial shedding after challenge

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ABSTRACT

Background: *Lawsonia intracellularis* causes porcine proliferative enteropathy and is one of the most economically important diseases in modern pig production worldwide. The Enterisol® Ileitis vaccine have been shown to reduce clinical disease and to increase weight gain, however, while the natural infection with *L. intracellularis* can provide complete protection against re-infection, this has not been achieved by this vaccine. We therefore undertook a detailed characterization of immune responses to *L. intracellularis* infection in vaccinated pigs (VAC) compared to previously infected pigs (RE) in order to pinpoint immunological determinants of protection.

Results: The VAC pigs shed *L. intracellularis* to the same extent as non-vaccinated pigs after challenge, however less *L. intracellularis* in ileum and lymph nodes was seen post mortem. In the RE group, challenge did not lead to *L. intracellularis* shedding and no challenge bacteria were found post mortem. In both VAC and RE the acute phase haptoglobin response was diminished and *L. intracellularis* specific IgG responses were delayed and reduced compared to non-vaccinated pigs. On the other hand *L. intracellularis* specific IFN- γ responses tended to develop faster in the VAC group compared to controls.

Conclusion: Although vaccinated and non-vaccinated pigs shed *L. intracellularis* at similar levels after challenge, a lower number of intestinal *L. intracellularis* was observed in the vaccinated pigs at post mortem inspection. This might be due to the observed faster CMI responses upon challenge in vaccinated pigs. Complete protection against infection without *L. intracellularis* shedding, however, was only seen after a previous infection resulting in IFN- γ production predominantly by CD8⁺ and CD4⁺ CD8⁺ cells. Improved protective vaccines against *L. intracellularis* should therefore target stimulation of these T cell subsets.

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1. Introduction

Lawsonia intracellularis is an obligate intracellular bacterium and the causative agent of proliferative enteropathy (PE) in pigs [1,2]. A commercial vaccine is available, and field trials have shown reduced clinical disease, increased daily weight gain and reduction in antibiotics consumption [3–5]. However, neither disease nor transmission of infection is prevented by vaccination, and thus *L. intracellularis* remains a major problem in the swine industry [6].

Abbreviations: qPCR, quantitative PCR; SAA, serum amyloid A; CMI, cell-mediated immune response; IFN, interferon; IHC, immuno-histo-chemistry.

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The immunological effect of the vaccine still remains to be elucidated. Guedes et al. [7] detected specific serum IgG from 5 to 13 weeks and specific IFN- γ response (measured by ELISPOT) 4–13 weeks post oral vaccination. Kroll et al. [8] found fewer lesions in the intestine, reduced faecal shedding and higher average daily weight gain in vaccinated pigs compared to non-vaccinated pigs after challenge with virulent *L. intracellularis*. The vaccinated pigs did not shed *L. intracellularis* bacteria or seroconvert before challenge, and a significantly higher proportion of non-vaccinated pigs shed *L. intracellularis* compared to the vaccinated pigs after challenge. Seroconversion occurred from 2 weeks post challenge, without significant differences in the pattern of seroconversion in the groups of vaccinated and non-vaccinated pigs [8].

Other studies have previously shown pigs with a primary virulent infection are protected against challenge with *L. intracellularis* without bacterial shedding after challenge [9,10] and reduced or

no *L. intracellularis* in tissue samples from the pigs re-inoculated at 12 weeks of age [10]. Serum IgG did not appear to correlate with protection while re-inoculated pigs responded to *L. intracellularis* challenge with a significantly increased IFN- γ response, which indicated induction of a cell-mediated immunity (CMI) memory recall response, with CD8⁺ and CD4⁺CD8⁺ double positive lymphocytes as main IFN- γ producers [11].

Here we present the outcome of *L. intracellularis* challenge infection in pigs vaccinated with Enterisol[®] ileitis (VAC) and compare acute phase protein responses, and humoral and CMI responses with non-vaccinated challenge control pigs (CC) and pigs protected against *L. intracellularis* re-infection (RE).

2. Materials and methods

2.1. Animals and experimental design

The experimental design has previously been presented in detail [10] (Experiment II) and responses to *L. intracellularis* infection for RE and CC groups partly presented [10,11]. In brief, standard crossbreed pigs, 5–6 weeks of age, received two ml oral vaccine (Enterisol[®] ileitis, Boehringer Ingelheim Vetmedica, Germany, batch 304–087 C with $10^{4.9}$ – $10^{6.1}$ tissue culture infective dose₅₀) according to the manufacturer's instructions (VAC group, $N=8$) at day 0. In parallel, a group of pigs was primarily infected by oral inoculation with approx. 10^9 *L. intracellularis* per pig via infected intestinal mucosa homogenate (RE group, $N=10$), and a group of pigs were held as non primary-infected challenge controls (CC group, $N=7$). All pigs were treated with antibiotics (tiamulin) day 21–28 and challenged at 12–13 weeks of age (day 49) with *L. intracellularis* (10^{10} bacteria per pig).

General condition and faeces texture was observed daily and individual observations that were different from normal observations in healthy pigs were noted. Individual blood- and faecal samples were collected once or twice a week from day -6 to 33, and 48 to 82. Weights were recorded at days 0, 21, 49, 70 and 84. Pigs were killed five weeks after *L. intracellularis* challenge.

All procedures of animal handling and experimentation were approved by the Danish Animal Experiments Inspectorate.

2.2. Quantification of *L. intracellularis* faecal shedding

DNA was extracted from 200 μ l samples of 1 gram faeces diluted $10\times$ in PBS by QIAcube[™] extraction robot and QIAamp DNA Stool Mini Kit (Qiagen, GmbH, Germany) [12] and the concentration was determined with Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Inc.).

For quantification of *L. intracellularis* DNA, a standard curve of purified *L. intracellularis* DNA was constructed and each qPCR experiment contained a known concentration of *L. intracellularis* DNA from the standard curve. Data (Ct values) from different experiments was transformed with RotorGene 6000 software 1.7 to DNA copies per gram faeces. The detection limit is 100 DNA copies per gram faeces [12].

2.3. Gross pathology and histological examinations

Gross pathological examination was performed to describe levels of PE and to exclude pigs with other lesions that may have influenced the experimental infection study.

Tissue samples were collected for IHC detection of *L. intracellularis* using routine procedures at DTU-Vet [13]. Blinded IHC examination was performed on tissue samples from ileum, mesenteric lymph nodes and colon, and scored: no staining, focal, moderate, or extensive detection of *L. intracellularis* antigen.

2.4. Measurement of acute phase proteins

Haptoglobin was analysed by a sandwich ELISA using the monoclonal antibody 3.8D7 directed against porcine haptoglobin as described [14]. Standard and samples were run in duplicate and the detection limit was 3.3 μ g/ml. A commercially available sandwich ELISA assay (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland) was used for determination of serum amyloid A (SAA). This assay is based on multi-species cross-reactive anti-human SAA monoclonal antibodies in a sandwich set-up as originally described by McDonald et al. [15]. Samples were tested according to the manufacturer's instructions. The detection limit of the assay was 31.25 μ g/ml.

2.5. Detection of *L. intracellularis*-specific IgG by ELISA

Measurements of *L. intracellularis*-specific IgG in serum samples were done by indirect ELISA with *L. intracellularis* deoxycholate (DOC) extract as previously described [16].

2.6. Measurement of cell-mediated immune responses

2.6.1. Whole blood IFN- γ release assay

As a measure of CMI response to *L. intracellularis*, a whole blood IFN- γ assay potentiated with porcine IL-18 was used [17]. The *L. intracellularis*-specific IFN gamma response to SDS extracted *L. intracellularis* antigen (SDS-Ag) was calculated after subtracting the background IFN- γ generated in the PBS culture for the respective sample. Based on a frequency distribution of IFN- γ levels in non-inoculated pigs a limit of unspecific response was set to 100 pg/ml. Six samples (of total 195 samples) were excluded as invalid due to high non-specific IFN- γ level in PBS cultures.

2.6.2. Cell culture and flow cytometry

Intracellular IFN- γ production, cell proliferation and T cell markers were measured by flow cytometry after culture of PBMCs with *L. intracellularis*-specific SDS-Ag, positive and negative controls, and potentiation or staining as outlined below. Intracellular IFN- γ production was measured in cultures containing rpoIL-18 (50 ng/ml) to potentiate IFN- γ production as previously described [11,17]. For measurement of cell proliferation, PBMCs prestained with CFSE at 1 μ M (Molecular probes) were cultured at 2×10^6 cells/ml in 24-well plates (Greiner-Bio One GmbH) for 5 days at 37 °C in 5% CO₂ in complete medium with SDS-Ag (5 μ g/ml), Con A (2 μ g/ml) or PBS. With each cell division, the intensity of CFSE staining will be halved and T cell proliferation was calculated as frequency of CFSE^{low} cells within gated cell population compared to intensity of parent population.

Cultured PBMCs were harvested and surface stained for CD4, CD8 α , and CD25 as outlined in Table 1. For CFSE stained cells, additional samples were used for exclusion of dead cells (7AAD⁺) and these samples were not stained for CD25.

Flow cytometric analyses were performed and analysed on a FACSCanto II with DIVA 6 software (BD Biosciences). Within a tight lymphocyte gate, approx. 30,000 and 70,000 cells were acquired for CFSE and IFN- γ samples, respectively.

IFN- γ producing cells within gated lymphocytes were identified and iMFI levels of IFN- γ responses calculated as frequency of IFN- γ producing cells \times median fluorescence intensity (MFI) of the cell population as previously described [11].

2.7. Statistics

One-way ANOVA with Bonferroni's post test was used to compare groups and Fisher's exact test used to compare numbers of responding pigs in the groups. Two-way repeated measures ANOVA

Table 1
Flow cytometry reagents.

Target	Secondary staining (IFN- γ)	Secondary staining (CFSE)	7-AAD
CD4 (74-12-4/IgG2b)-biotin ^a	Streptavidin-PerCP ^d	Streptavidin-PerCP ^d	Streptavidin-APC ^d
CD8 α (76-2-11/IgG2a) ^a	Goat-anti mouse IgG2a-FITC ^c	Goat-anti mouse IgG2a-AF647 ^c	anti-IgG2a-PE ^c
CD25 (K231.3B2/IgG1)	Goat-anti mouse IgG1-PE ^c	Goat-anti mouse IgG1-PE ^c	NA
IFN- γ (cc302/IgG1)-AF647 ^b	–	NA	NA
Negative control (IgG1)-AF647 ^b	–	NA	NA
Live-dead	NA	NA	7-AAD ^e

^a DTU-Vet produced mAb.^b AbD Serotec, Oxford, UK.^c Southern Biotech, Alabama, USA.^d BD Bioscience, Broendby, Denmark.^e Sigma Aldrich, Broendby, Denmark.

with Bonferroni post test was used to compare the development of IgG response. A *t*-test was used to compare differences when only two groups were compared.

2.8. Results

Faecal shedding, histopathology, haptoglobin, IgG and IFN- γ responses for the pigs in RE and CC groups have been described in detail previously [10,11].

3. Responses after *L. intracellularis* vaccination or primary infection

3.1. Clinical observations

All pigs had normal appetite, faecal texture and showed no signs of reduced well-being, except for two (nos. 37 and 52) of the 10 pigs receiving a primary *L. intracellularis* infection which presented soft faeces, but normal general condition in the period after inoculation.

3.2. *L. intracellularis* faecal shedding

Lawsonia shedding in faeces was not observed in vaccinated or CC groups at any time point after primary inoculation by routine PCR diagnostics or qPCR (day 18) (data not shown). In contrast all primarily inoculated pigs (RE group) shed *L. intracellularis* [10] and qPCR (day 18) revealed *L. intracellularis* shedding at a range of 10^5 – 10^8 *L. intracellularis* DNA copies/g faeces in these pigs.

3.3. Acute phase protein responses

The area under curve (AUC) at day 6–26 of haptoglobin responses after vaccination in the VAC group was not significantly increased compared to the non-vaccinated CC group, whereas the primarily infected pigs (RE-group) showed significantly increased haptoglobin responses ($P < 0.05$) (Fig. 1A). Serum amyloid A (SAA) levels were characterized by single peaks of increased SAA response in the pigs within day 6–26. In this period increased SAA responses (peak responses > 100 μ g/ml) were found in 6 of 8 VAC-pigs and in 9 of 10 primarily infected pigs (RE-group), whereas only in one of seven non-vaccinated pigs (CC-group) (Fig. 1B). Peak SAA responses were mainly identified at day 14 or 18 (eight RE pigs and three VAC pigs) whereas in three VAC-pigs peak SAA responses were identified at day 6 or 11. Only one CC pig showed peak SAA responses $>$ background level at day 6.

3.4. Antibody and CMI responses

The oral vaccination did not induce measurable levels of antigen-specific IgG in serum samples in the period after vaccination (Fig. 2A). Some pigs, in all groups had maternal antibodies, which decreased within the first 2–3 weeks of the experiment.

Nor did the oral vaccination induce IFN- γ levels above background level (100 pg/ml). Single outliers with apparent *L. intracellularis* antigen-specific IFN- γ responses were observed in both VAC and CC groups (Fig. 2B). In comparison, the IFN- γ responses in age-matched *L. intracellularis* subclinical infected pigs were moderate with around 50% of the pigs responding with antigen-specific IFN- γ above background level (> 100 pg/ml), and with several pigs showing a sustained high antigen-specific IFN- γ response (> 1000 pg/ml) even at day 48 p.i. [11].

4. Responses after *L. intracellularis* challenge

4.1. Clinical observations and weight gain

Before and after *L. intracellularis* challenge at 12–13 weeks of age, all pigs had normal appetite, faecal texture and showed no signs of reduced well-being. Mean daily weight gain in the whole study period was not statistically different between the VAC Group and the non vaccinated CC Group or the RE Group.

4.2. *L. intracellularis* faecal shedding

Vaccinated and non-vaccinated CC-pigs shed high levels of *L. intracellularis* from 6 days after challenge, without significant differences in level of faecal shedding between vaccinated and non-vaccinated CC pigs at day 6 ($P = 0.22$), 14 ($P = 0.18$) or 18 ($P = 0.26$) post challenge (Fig. 1D), or in numbers of shedding pigs (data not shown). In contrast, no shedding was seen at any time after challenge in the pigs previously infected with *L. intracellularis* (RE) [10].

4.3. Immunohistopathology

In two (nos. 33 and 48) of 8 vaccinated pigs focal detection of *L. intracellularis* was found in ileum, and in lymph nodes (no. 33). In contrast *L. intracellularis* was moderate to extensively detected in ileum and lymph nodes in 4 of 7 non-vaccinated pigs (CC), whereas *L. intracellularis* was not detected in the 10 re-inoculated pigs (RE).

4.4. Acute phase protein responses

Comparing the haptoglobin AUC responses day 55–75, i.e. 6–26 days post challenge (Fig. 1C), a significantly reduced haptoglobin response was observed in the RE-group compared the CC-group ($P < 0.001$), but not compared to the VAC-group. Although the VAC-group was not significantly different compared to the CC group, the lack of difference from the RE-group indicates a tendency to reduced haptoglobin response compared to the non-vaccinated CC-group. The low haptoglobin response may indicate a general reduced infection/disease in the group of vaccinated pigs compared to the group of non-vaccinated pigs.

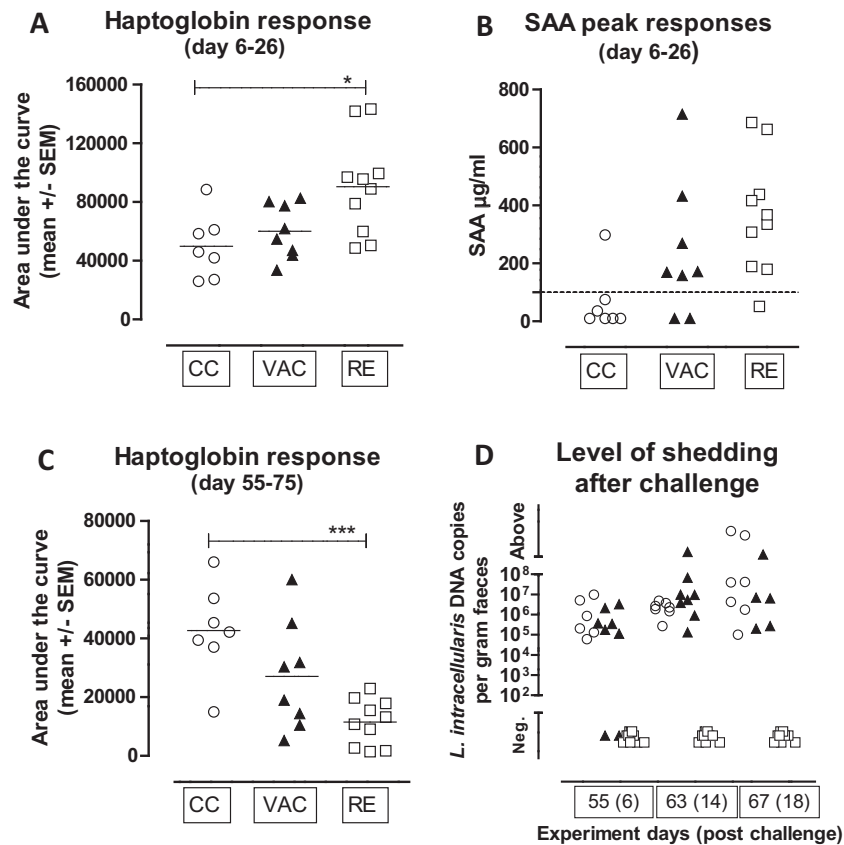


Fig. 1. Acute phase protein responses after vaccination, primary infection and challenge, and level of *L. intracellularis* faecal shedding after challenge. (A) Haptoglobin responses (area under the curves (AUC)) day 6–26 after vaccination (VAC), *L. intracellularis* primary inoculation (RE) or without treatment (CC). (B) Peak SAA responses in the same period. Dotted line indicates background level of SAA (100 µg/ml). (C) Haptoglobin responses (AUC) in the pig groups day 55–75, i.e. day 6–26 after challenge at day 49. (D) Levels of *L. intracellularis* measured by quantitative PCR (qPCR), i.e. DNA copies per gram faeces, on experiment day 55, 63, and 67, corresponding to days 6, 14 and 18 after challenge, in CC (circles), VAC (black triangles) and RE (squares). Due to inadequate sample, data from a few pigs is missing (day 55: 1 CC pig, day 67: 3 VAC pigs).

4.5. Antigen-specific humoral and cell-mediated immune responses after *L. intracellularis* challenge

4.5.1. Serum antibody response

After challenge *L. intracellularis*-specific serum IgG responses developed more slowly and peak levels in the vaccinated pigs were significantly reduced compared to the responses in the non-vaccinated CC-pigs (Fig. 2A, $P < 0.001$). At 11 days post challenge, positive response were only measured in 2 of 8 VAC-pigs compared to 5 of 7 non-vaccinated pigs, and at day 14 post challenge all 7 non-vaccinated pigs reached app. 50% of peak level (50 OD%) whereas 7 of 8 VAC-pigs showed responses below 50 OD% ($P < 0.01$).

4.6. CMI response

After challenge, *L. intracellularis*-specific IFN- γ responses increased in vaccinated and non-vaccinated CC-pigs (Fig. 2B). The IFN- γ response tended to be earlier in the vaccinated pigs with near significant higher level of IFN- γ response in the VAC-group at day 60 (11 days post challenge) than in the CC-group ($P = 0.062$).

L. intracellularis-specific T cell proliferation was measured within cell subsets as shown in Fig. 3A. All samples cultured with Con A, as positive control, showed high level of T cell proliferation in the three cell subsets compared to PBS culture. Proliferating cells expressed CD25. All pigs showed increased antigen-specific T cell proliferation compared to before challenge (Fig. 3C). At this time all VAC-pigs and CC-pigs showed antigen-specific proliferation at background levels, whereas the RE-pigs, which had experienced a previous *L. intracellularis* infection, showed significantly

increased responses, with the highest proliferation rate observed in CD4⁺CD8⁺ cells. Peak responses of antigen-specific proliferation were observed at day 18 or 26 post challenge with a decline at day 33 post challenge. The RE-group showed increased antigen-specific proliferation within CD4⁺CD8⁺ cells and CD8^{high}CD4^{neg} cells compared to VAC-group, which in turn showed responses comparable with the responses in the non-vaccinated CC-group.

By intracellular IFN- γ staining, *L. intracellularis*-specific IFN- γ response above background level (iMFI > 15) was identified in three of eight VAC-pigs at 26 days post challenge (Fig. 4). In these pigs a CD4⁺CD8^{neg} phenotype of IFN- γ producing cells was more pronounced compared to phenotypes of IFN- γ producing cells in the RE pigs, which were mainly CD4⁺CD8⁺ and CD8⁺CD4^{neg} (Table 2). Profiles of IFN- γ producing cells for CC-pigs and RE-pigs on other time points was presented by Cordes et al. [11].

5. Discussion

In field studies, vaccinated pigs have shown enhanced daily weight gain compared to non-vaccinated pigs following *L. intracellularis* infection, and with reduced consumption of antibiotics in the vaccinated pigs [5]. In the present study pigs receiving the oral attenuated *L. intracellularis* vaccine strain neither developed antibodies nor a CMI response in the period after vaccination, and did not show reduced shedding following a challenge inoculation with a field strain. However, upon challenge we observed vaccinated pigs had significantly reduced acute phase protein responses and lower levels of *L. intracellularis* at post mortem IHC examination compared to naive age-matched control pigs indicating the vaccine strain was

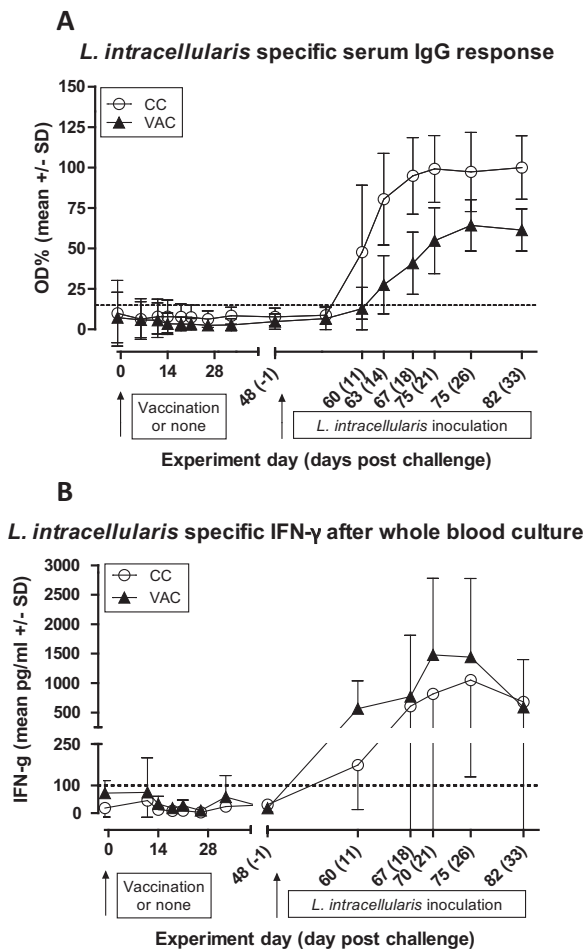


Fig. 2. *L. intracellularis*-specific immune IgG and CMI responses. Development of *L. intracellularis*-specific IgG (A) and whole blood IFN- γ (B) responses in the VAC-group and CC-group. Dotted line indicates cut-off level at 15 OD% and 100 pg/ml, respectively.

inducing some kind of response in the vaccinated pigs. The *L. intracellularis* vaccine strain has been attenuated by several cell culture passages [8], and differ in transcriptional profiling of a large number of genes compared to pathogenic *L. intracellularis* during *in vitro* infection [18]. The different virulence phenotype between attenuated bacteria in the vaccine and pathogenic field strains is, probably together with differences in dose [19], likely responsible for this difference in protection against re-infection [9,10]. With the obligate intracellular nature and multiplication of *L. intracellularis* in the cytoplasm [20], it is reasonable to speculate that a CMI host response is needed to efficiently control this pathogen. Previously, Guedes et al. have reported *L. intracellularis*-specific IFN- γ response by ELISPOT in pigs inoculated with the attenuated vaccine strain [7] although only 2 of 10 vaccinated pigs and 4 of 10 pigs inoculated with pathogenic bacteria (inoculum 4.4×10^9 bacteria per pig) showed consistent IFN- γ response with wide variance among the ELISPOT results for the other pigs. In contrast to the present study, Guedes et al. also showed seroconversion and PCR detection of *L. intracellularis* in faecal samples after inoculation with the vaccination strain. While vaccinated pigs in the current study did not show seroconversion, induction of CMI responses by IL-18 potentiated whole blood IFN- γ assay (although a priming of CMI responses was observed as discussed below) or shed bacteria after vaccination, vaccinated pigs did show an induction of acute phase protein responses (haptoglobin and/or SAA), with a similar kinetic profile (data not shown) as for pigs primarily infected with

L. intracellularis. These acute phase protein responses were most likely induced by the attenuated *L. intracellularis* vaccine strain as the vaccine is without adjuvant. Haptoglobin is a slow acute phase protein with a rather protracted response after *L. intracellularis* infection [10] and the optimal way to represent the magnitude of this acute phase protein response during this infection is to derive AUC rather than to look at peak responses. In contrast, the acute phase response of SAA, as a very quickly reacting porcine acute phase protein, also normalizing very quickly at the end of the insult [21], is more faithfully represented by its peak value. SAA has previously been measured in pigs infected with swine influenza virus (SIV), *Pasteurella multocida* [22], porcine Circovirus type 2 (PCV2) infection [23], and after *Brachyspira hyodysenteriae* induced diarrhoea [24] with SAA serum levels comparable to what was measured in subclinical *L. intracellularis* inoculated or vaccinated pigs of same age in the present study.

These differences in immunological response to attenuated *L. intracellularis* could be a result of the vaccine dose. Enterisol ileitis vaccine contains $10^{4.9}$ – $10^{6.1}$ TCID₅₀ per vaccine dose and while we administered a controlled oral vaccine dose to each individual pig, in the study by Guedes et al. the pigs were exposed to the attenuated vaccine strain via drinking water allowing for different individual dosing [7]. Nogueira et al. demonstrated dose-dependency of local and systemic responses of *L. intracellularis*-specific IgG and IgM, as well as TNF- α and TGF- β 1 with 10 \times compared to 1 \times dose of Enterisol[®]Ileitis [19]. Nogueira et al. also observed a dose-dependent reduction in level of faecal *L. intracellularis* shedding after experimental challenge with infected intestinal mucosa inoculum (10^9 *L. intracellularis* per pig) to pigs vaccinated with Enterisol[®]Ileitis. Thus the number of attenuated *L. intracellularis* seemed to affect the ability to induce protection against *L. intracellularis* colonisation/shedding. In our experiment the level of *L. intracellularis* shedding post challenge (mean of day 6, 14 and 18) was 2.0×10^7 and 8.5×10^7 bacteria/g faeces in VAC- and CC-pigs, respectively. This was comparable to the level of shedding in the RE-pigs after the primary *L. intracellularis* infection, although primary dose of inoculum was lower and the pigs younger.

We have previously shown that re-infection of pigs with field strain of *L. intracellularis* (infected intestinal mucosa inoculum) boosts the *L. intracellularis*-specific IFN- γ responses and development of specific CD8⁺ cells and CD4⁺CD8⁺ cells after challenge. In the present study, pigs vaccinated with attenuated *L. intracellularis* exhibited a trend towards faster development of IFN- γ responses after challenge compared to the non-vaccinated CC-pigs, which could indicate the vaccine also primes a CMI response. Phenotypic profiling of the *L. intracellularis*-specific proliferating cells and of IFN- γ producing cells indicated, however, a more pronounced response in CD4⁺CD8^{neg} cells in the vaccinated pigs compared to the profiles of IFN- γ producing cells in the RE-pigs. As *L. intracellularis* escape and multiply in the cytosolic department of the host cell, antigen-specific immune mediated protection is likely mediated through MHC class I presentation to CD8⁺ cytotoxic T cells, and the difference in priming of CD8⁺ T cells may thus contribute to the explanation of the lesser level of protection after vaccination compared to prior exposure to virulent field strains. Replication of *L. intracellularis* takes place in the intestines, and an optimal study design for detailed studies of protective immunity should thus include intestines and local draining lymph nodes. For a number of practical reasons this was not possible in the current study. Interestingly, the development of serum IgG antibodies was delayed in vaccinated compared to CC pigs, indicating antibodies may reveal level of infection and thus act as surrogates of infection rather than correlates of protection in *L. intracellularis*.

In conclusion the presented study indicate priming of a cell-mediated immune response in pigs vaccinated with Enterisol[®]Ileitis when challenged with *L. intracellularis*. Primed CMI responses

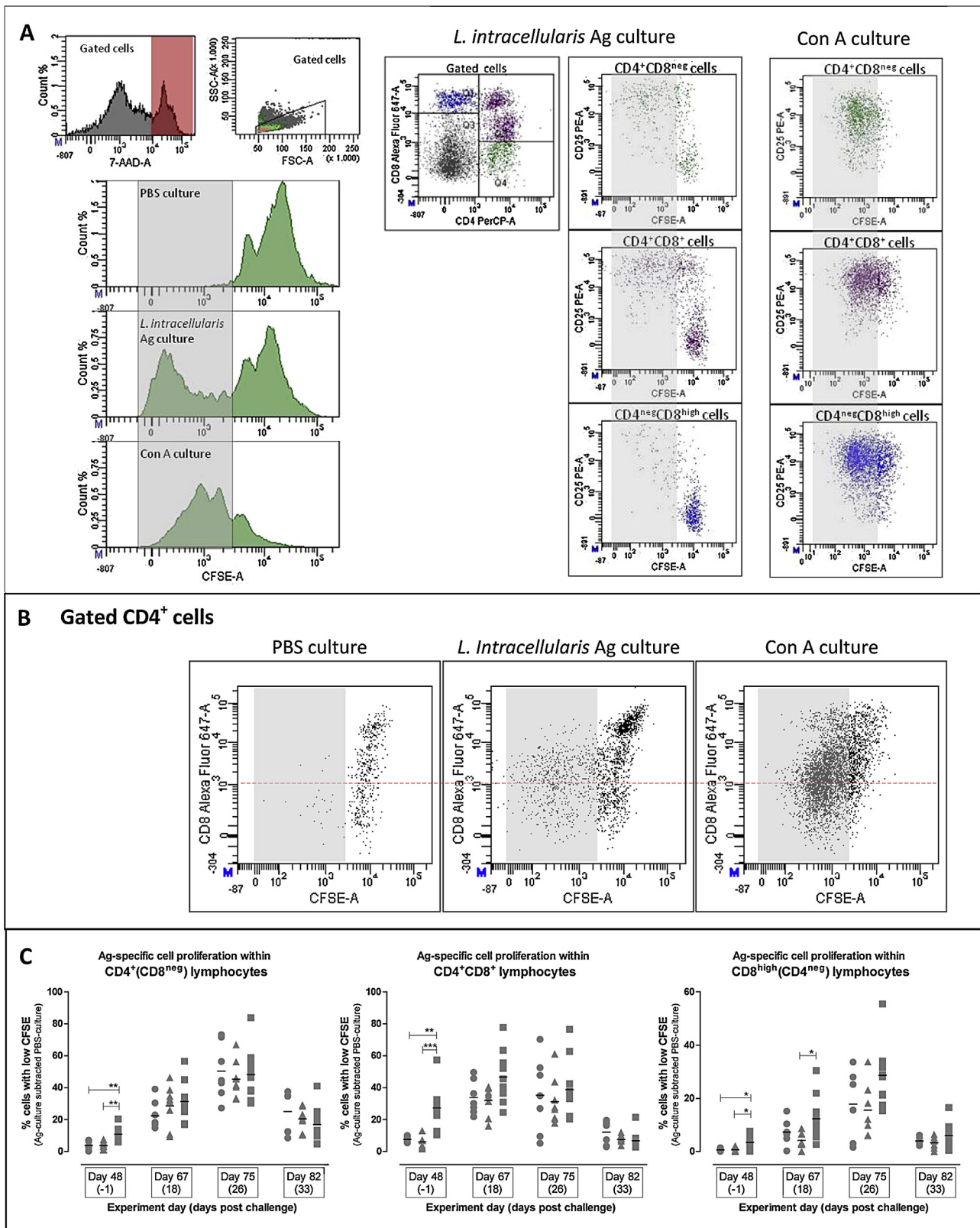


Fig. 3. *L. intracellularis*-specific T cell proliferation after challenge. (A) The flow cytometry gating strategy with one pig (VAC no. 33, day 75) as an example. Cells were gated in FSC/SSC plot excluding cell profiles with high 7AAD staining (expected dead cells). Histogram plots show CFSE fluorescence intensity in gated cells from cultures with PBS, Ag and Con A. Grey field indicate proliferating cells (reduced CFSE intensity compared to background in PBS-culture). Subpopulations within gated cells were identified in CD4 versus CD8 plot. Proliferation within cell subsets are shown for CD4⁺CD8^{neg} cells, CD4⁺CD8⁺ cells and CD4^{neg}CD8^{high} cells in *L. intracellularis* Ag- culture and Con A-culture as positive control (plots showing CFSE versus CD25). Cells with low CFSE fluorescence intensity were also found within the CD4^{neg}CD8^{neg} cell population; these were mainly CD25 negative (data not shown). (B) CD8 α expression versus CFSE proliferation in total CD4⁺ cells within gated lymphocytes for PBS, *Lawsonia* and Con A cultures of the same sample as 3 (A). (C) The *L. intracellularis*-specific proliferation within CD4⁺CD8^{neg} cells, CD4⁺CD8⁺ cells and CD4^{neg}CD8^{high} cells based on CD25 stained samples. Mean as well as scattered individual data points are shown for CC (circles), VAC (triangles) and RE (squares) at indicated time points. Test for significant differences was performed by unpaired *t* test (* *P* < 0.05, ** *P* < 0.01).

Table 2
Phenotype profiles and relative distribution of *L. intracellularis* specific IFN- γ producing cells.

Group	n/N ^a	Relative distribution (%) of IFN- γ producing cells (mean \pm SEM)			Residual (other cells)
		CD4 ⁺ CD8 α ^{neg}	CD4 ⁺ CD8 α ⁺	CD4 ^{neg} CD8 α ^{high}	
CC	1/7	8.0	45.3	37.6	9.0
VAC	3/8	15.8 \pm 0.7	29.2 \pm 0.3	41.5 \pm 2.2	13.6 \pm 1.7
RE	8/10	9.2 \pm 1.9	35.1 \pm 3.2	40.8 \pm 5.6	14.9 \pm 3.1

^a Number of pigs within the group showing *L. intracellularis* antigen-specific IFN- γ response (iMFI > 15) at day 75 (day 26 post challenge).

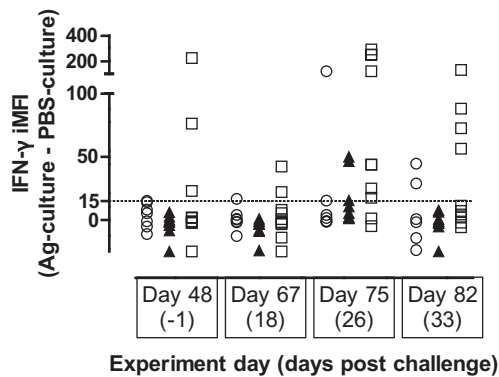


Fig. 4. Identification of pigs showing *L. intracellularis*-specific IFN- γ response detected by intracellular flow cytometry. *L. intracellularis*-specific IFN- γ responses (iMFI in Ag-cultures subtracted iMFI in PBS-cultures) in CC (circles), VAC (black triangles) and RE (squares) at the indicated days. For pigs with IFN- γ responses iMFI > 15 the relative distribution of IFN- γ producing cells was calculated and shown in Table 2 for day 75 at which time point three pigs in the VAC-group showed IFN- γ iMFI > 15.

were observed by faster development of antigen-specific IFN- γ response in whole blood assay, and detection of antigen-specific IFN- γ producing cells as well as altered antigen-specific T cell proliferation after challenge. Although vaccination did not inhibit shedding of *L. intracellularis* compared to non-vaccinated pigs, an increased acute phase protein response was identified following vaccination, whereas a reduced haptoglobin response was measured after challenge, indicating that the vaccinated pigs were less affected by the challenge inoculation than the non-vaccinated pigs.

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Conflict of interest statement

The authors declare that they have no competing interests.

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