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Immune responses following neonatal vaccination with conserved F4 fragment of VtaA proteins from virulent *Glaesserella parasuis* adjuvanted with CAF®01 or CDA

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ABSTRACT

Glaesserella parasuis is a Gram-negative bacterium that colonizes the upper airways of swine, capable of causing a systemic infection called Glässer's disease. This disease is more frequent in young post-weaning piglets. Current treatments against *G. parasuis* infection are based on the use of antimicrobials or inactivated vaccines, which promote limited cross-protection against different serovars. For this reason, there is an interest in developing novel subunit vaccines with the capacity to confer effective protection against different virulent strains. Herein, we characterize the immunogenicity and the potential benefits of neonatal immunization with two different vaccine formulations based on the F4 polypeptide, a conserved immunogenic protein fragment from the virulence-associated trimeric autotransporters of virulent *G. parasuis* strains. With this purpose, we immunized two groups of piglets with F4 combined with cationic adjuvant CAF®01 or cyclic dinucleotide CDA. Piglets immunized with a commercial bacterin and non-immunized animals served as control groups. The vaccinated piglets received two doses of vaccine, at 14 days old and 21 days later. The immune response induced against the F4 polypeptide varied depending on the adjuvant used. Piglets vaccinated with the F4+CDA vaccine developed specific anti-F4 IgGs, biased towards the induction of IgG1 responses, whereas no anti-F4 IgGs were *de novo* induced after immunization with the CAF®01 vaccine. Piglets immunized with both formulations displayed balanced memory T-cell responses, evidenced upon *in vitro* re-stimulation of peripheral blood mononuclear cells with F4. Interestingly, pigs immunized with F4+CAF®01 controlled more efficiently a natural nasal colonization by a virulent serovar 4 *G. parasuis* that spontaneously occurred during the experimental procedure. According to the results, the immunogenicity and the protection afforded by F4 depend on the adjuvant used. F4 may represent a candidate to consider for a Glässer's disease vaccine and could contribute to a better understanding of the mechanisms involved in protection against virulent *G. parasuis* colonization.

Introduction

Glaesserella (formerly *Haemophilus*) *parasuis* is a Gram-negative gammaproteobacterium member of the *Pasteurellaceae* family that colonizes the upper respiratory tract of pigs early after birth [1]. *G. parasuis* comprises strains that can be pathogenic to pigs, espe-

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cially in the post-weaning period. At that age, around 4–8 weeks of life, susceptible piglets may develop invasive *G. parasuis* infection leading to Glässer's disease, which is characterized by fibrinous polyserositis, arthritis and meningitis. Glässer's disease causes serious economic and welfare problems in the swine industry [2]. *G. parasuis* infection can be treated using antimicrobials, but the increasing concern about the use of these compounds makes vaccination the main alternative tool to control the impact of this infection [3].

Neonate vaccination is an effective approach for the protection against infectious diseases that are common in the early stages of life [4]. However, newborn animals have an immature immune system, and protective immunity in these early stages of life can be difficult to achieve due to the immune system's skew towards stimulation of Th2 and Treg responses, with limited Th1 and antibody responses [5,6]. Moreover, neonate immunity is also affected by other factors such as maternal immunity components acquired via colostrum that, in the case of pigs, can modulate the immune maturation of the offspring and interfere with vaccination [7–9], and the microbiota that colonize their mucosae [10,11].

Current vaccines against Glässer's disease consist of autogenous or commercial bacterins, which confer protection against homologous or a narrow heterologous range of serovars [12]. The lack of cross-protection afforded by these vaccines urges to look for broad-spectrum strategies that could protect against heterologous virulent serovars. In this respect, subunit vaccines consisting of surface-exposed proteins or protein domains exclusively conserved in pathogenic strains of *G. parasuis* represent an attractive alternative. Among possible candidates, the virulent-associated trimeric autotransporters (VtaA), a family of outer-membrane proteins of *G. parasuis* involved in adhesion to extracellular proteins and phagocytosis resistance by alveolar macrophages, may be of interest [13,14]. Comparison of the amino acid sequence among the VtaAs from strains with different degrees of virulence allowed the identification of a surface-exposed fragment, named F4, within the group 1 and 2 VtaAs from the Nagasaki strain (serovar 5) that is highly conserved in virulent *G. parasuis* [15]. Recent work performed in our laboratory has additionally shown that sow immunization with F4 emulsified with Carbopol Polymer adjuvant not only induced anti-F4 specific immune responses but also modulated the immunity traits in their offspring with an increase of circulating TGF-β [16].

Here we explored the vaccine potential of the F4 protein fragment in neonate pigs, testing the efficacy of two novel adjuvants: the Cationic Adjuvant Formulation 01 (CAF®01) and the bis-(3,5)-cyclic dimeric adenosine monophosphate (CDA), specifically designed to stimulate different immune pathways. CAF®01 is a liposome-based mixture of the cationic surfactant N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) and the synthetic glycolipid analogue to the *Mycobacterium tuberculosis* cord factor α,α'-treha lose 6,6′-dibehenate (TDB), and it was chosen in this study due to its ability to promote Th1/Th17-like responses [17,18]. Furthermore, CAF®01 has been shown to be efficient in the induction of immunity in neonates [19–22]. CDA is a monocyclic dinucleotide naturally secreted by *Listeria monocytogenes*, which was selected due to its ability to efficiently activate STING, the "Stimulator of Interferon Genes", triggering antibody production as well as balanced Th1/Th2/Th17-like responses and induction of cytotoxic CD8+ lymphocytes [23,24]. Recent *in vitro* and *in vivo* studies suggested that CDA is also a suitable candidate for the development of neonatal vaccines [25].

In this vaccination study, we assessed the humoral and cell-mediated immune responses in neonate piglets following immunization with the two adjuvanted F4 formulations. In addition, the effect of the vaccines on the natural colonization by virulent *G. parasuis* was also examined.

Material and methods

Ethics regulation

Animal experiments were conducted in AM Animalia (La Vall de Bianya, Girona, Spain) according to the ARRIVE guidelines, approved by the AM Animalia Ethics Committee for Animal Experimentation with number CEEA 20/20-P1 and CEEA 20/20-P2 in compliance with the EU directive 63/2010, the Spanish legislation (RD 53/2013) and the Catalan law 5/1995 and decree 214/1997.

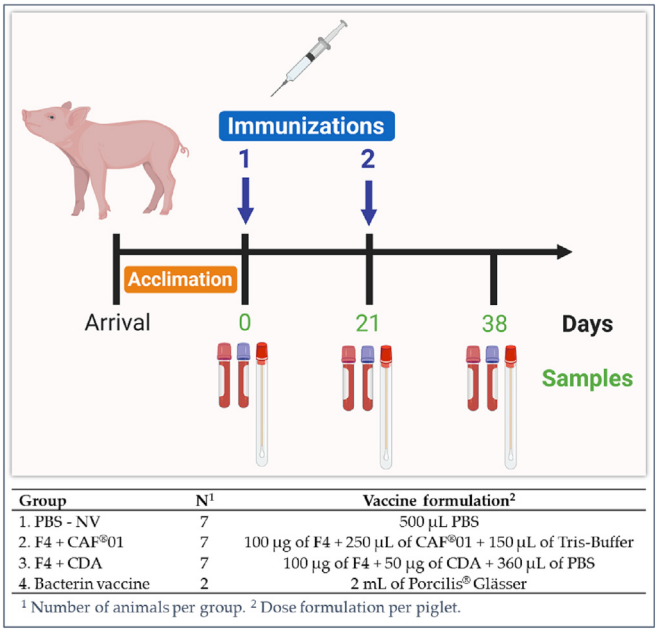
Experimental vaccines preparation

The conserved F4 antigen from VtaAs consisting of the sequence AGPTGNSELKGITSIANGNDATKANGAKITLSAGSTD KTVNVNDAKITN VAAGTADTDAVNVSQLNTKAAASKTEVEAGKNVKVTSKTGANGQNIYN VSVSGDLSDITSISNGDTKVSLGKDKQGNPVVNMNGARITNVGDGSAE GDIVNVRQLNKVVSSVNTGFNQLSRDIGRSARGS was produced as a His-tagged recombinant protein by induction of the expression plasmid pASK-IBA33plus-F4 in *E. coli* BL21. Briefly, transformed bacteria were grown overnight at 37 °C in LB broth supplemented with 100 μg ampicillin and induced in the stationary phase with AHT 0.2 μg/mL to allow the optimal expression of F4. For purification, bacterial cultures were pelleted and later resuspended in saline sodium phosphate buffer with 1 μM Pefabloc®SC (Sigma-Aldrich, Madrid, Spain) and disrupted with a Branson 450 Digital Sonifier (Branson Ultrasonics Corporation, Brookfield, CT, USA) using 50 pulses of one second in an amplitude of 20%. Purification of His-F4 was performed with His-Spin Trap columns (GE Healthcare Life Sciences, Chicago, IL, USA) with the help of 20 mM of imidazole and later dialyzed against PBS. F4 purity was confirmed by protein gel electrophoresis and Coomassie blue staining and later quantified with the Pierce BCA Protein Assay Kit (ThermoFisher, Madrid, Spain). Purified F4 was stored at −20 °C until use.

CAF®01 and CDA were provided by the *Statens Serum Institut* (Copenhagen, Denmark) and the *Helmholtz-Zentrum für Infektionsforschung* (Braunschweig, Germany), respectively. Experimental vaccine formulations were prepared and administered in seven pigs for each group (Fig. 1), injecting 100 μg of the immunogen in a total volume of 500 μL per dose intramuscularly in the neck of the piglets. Two additional piglets were immunized with the Porcilis® Glässer vaccine, a commercial product composed of inactivated *G. parasuis* serovar 5 adjuvanted with dl-α-tocopheryl acetate, as controls for the *in vivo* assay, following the recommendations of the manufacturer (MSD Animal Health, Salamanca, Spain).

Experimental design

The study design is displayed in Fig. 1. Twenty-three Landrace × Duroc piglets of about five days-of-age were included in the study. Piglets were obtained from 8 sows between parities 3–5 with variable antibody values against *G. parasuis* according to the commercial Ingezim–Haemophilus ELISA (Ingenasa, Madrid, Spain) and low OD values measured by an *in-house* ELISA against the F4 fragment (see F4-specific antibody detection). Animals were transported to the experimental facilities of AM Animalia (La Vall de Bianya, Girona, Spain), where they were housed in one room, and distributed into four pens, one group in each pen. Each group included piglets from all the sows from which they were weaned. Animals were fed *ad libitum* during all the experiment and were treated with 5 mg/kg of ceftiofur (Naxcel porcino, Zoetis S.L.U., Spain) during the first week of the study to prevent undesirable bacterial diseases. After seven days of acclimation, animals were intramuscularly vaccinated in the left flank of the neck on day 12



Group	N[1]	Vaccine formulation[2]
1. PBS - NV	7	500 µL PBS
2. F4 + CAF®01	7	100 µg of F4 + 250 µL of CAF®01 + 150 µL of Tris-Buffer
3. F4 + CDA	7	100 µg of F4 + 50 µg of CDA + 360 µL of PBS
4. Bacterin vaccine	2	2 mL of Porcilis® Glässer

[1] Number of animals per group. [2] Dose formulation per piglet.

Fig. 1. Schematic representation of the *in vivo* study procedure and group distribution of the experimental vaccines. Piglets from 3 to 4 days of age from 8 sows were transported to the animal facilities and left for acclimation for one week. Each group included piglets collected from every sow at the beginning of the study. After acclimation, piglets were twice immunized 21 days apart. Samples of clotted blood, EDTA-treated blood and nasal swabs were collected on days 0 (prior to vaccination), 21 (after the first immunization, prior to boosting) and 38 (17 days after boosting). NV: non-vaccinated. Figure created with BioRender.com.

of life (Day zero of the study; D0) and twenty-one days later, piglets were boosted with a second dose of each vaccine (D21), this time in the right flank of the neck. Whole blood (EDTA), sera and nasal swabs were taken on study days D0, D21 and D38 (17 days after boosting). Sera obtained by centrifugation (10 min at 860 × *g*) of clotted-blood tubes were aliquoted and stored at −80 °C. Nasal swabs were resuspended in 500 µL of PBS and stored at −80 °C until use.

DNA extraction and G. parasuis detection by PCR

Two hundred microlitres of the resuspended nasal swabs were processed with the MagMax Pathogen kit (Life Technologies, Madrid, Spain) according to the manufacturer's instructions. The presence of virulent and non-virulent *G. parasuis* colonizing strains was monitored by PCR using two primer sets that differentially amplify their *vtaA* leader sequences (LS-PCR) [26]. Four µL of the DNA extracted from the nasal swabs were used as a template for the reaction. Purified DNA from the virulent Nagasaki (serovar 5) and the non-virulent SW114 (serovar 3) strains were included as controls. Molecular serotyping was performed by a serotype-specific PCR using the DNA purified from nasal swabs as template [27], to assess the serovars of naturally colonizing *G. parasuis*.

G. parasuis and F4-specific antibody detection

The commercial Ingezim-Haemophilus ELISA (Ingenasa, Madrid, Spain) was used for the detection of total antibodies against *G. parasuis*, following the manufacturer's indications. In addition, an *in-house* ELISA was used for detection of antibodies against F4 [16]. Briefly, high binding plates were coated overnight at 4 °C with 500 ng of F4 per well. After washes, wells were blocked with 1% casein in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-Tw20). Sera and nasal swab resuspensions were serially diluted in blocking solution or used undiluted. After a 1 h of incubation at 37 °C, wells were incubated with a goat anti-porcine IgG HRP-conjugated antibody (Sigma-Aldrich, Madrid, Spain) diluted 1:10,000. For IgG1 and IgG2 specific detection, a mouse anti-pig IgG1 or IgG2 (both from Bio-Rad Laboratories, Hercules, CA, USA) were used diluted 1:2,000, followed by a goat anti-mouse IgG conjugated with HRP (Sigma-Aldrich, Madrid, Spain) diluted 1:10,000. To detect specific IgM and IgA responses, goat anti-porcine IgA or IgM HRP conjugated antibodies (both from AbD Serotec, Oxford, UK) were both used diluted 1:1,000. Positive reactions in all the ELISAs were developed using the 3,3,3,5-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, Madrid, Spain) and the reactions were stopped with 1 N sulfuric acid. Plates were then read in a Power Wave XS spectrophotometer (Biotech, Winooski, VT, USA) at 450 nm.

PBMC isolation and specific IFN-γ ELISPOT

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 mL of EDTA-treated blood of D0 control pigs (prior to PBS or Porcilis® Glässer injection) to determine the presence of F4 specific IFN-γ secretory T-cells by ELISPOT at the starting point. Additional IFN-γ ELISPOT assays were later performed using PMBCs from all animals at 21 and 38 days postvaccination (D21 and D38). PBMC isolation was performed under a density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, Madrid, Spain), followed by an osmotic shock to remove red blood cells. Concentration of PBMCs was adjusted and 5 × 10^5 cells/well were plated in flat-bottomed 96-well tissue culture plates with RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy), 1% glutamine (Gibco Life Technologies, Madrid, Spain), 1% penicillin-streptomycin (Gibco Life Technologies, Madrid, Spain) and 0.05 mM of β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain). PBMCs were freshly used to perform an IFN-γ Enzyme-Linked ImmunoSpot Assay (ELISPOT), following a protocol described before [28], with minor modifications. Briefly, high binding 96-well plates (Costar Corning Incorporated, New York, NY, USA) pre-coated with 250 ng of porcine anti-IFN-γ per well (Clone P2G10 Mouse IgG1, BD Pharmingen™, San José, CA USA) were used to plate PBMCs. PBMCs were stimulated for 48 h at 37 °C and 5% CO_2 with F4 (2 µg/mL) to detect the F4 specific IFN-γ secretory cells. Phytohemagglutinin (10 µg/mL, Sigma-Aldrich, Madrid, Spain) and RPMI were used as positive and negative control, respectively. After 48 h of incubation, plates were washed to remove cells and stained with 25 ng of biotinylated anti-IFN-γ antibody (Clone P2C11 Mouse IgG2a, BD Pharmingen™, San José, CA USA) and developed with HRP-streptavidin (Invitrogen Life technologies. Madrid, Spain) followed by insoluble TMB (Merck Life Science, Madrid, Spain). Resulting spots were counted under the microscope (Stereoscopic Zoom Microscope SMZ800, Nikon Instruments Inc., Chiyoda, Japan). In the analysis, background spots obtained in the control wells (stimulated with RPMI alone) from each animal, were subtracted to the F4-stimulated ones and expressed by 10^6 cells for statistical analysis.

Immune phenotyping and cytokine detection

In parallel to the IFN-γ ELISPOT, PBMCs were plated on flat-bottomed 96-well cell culture plate (SPL Biosciences, Gyeongido, Korea) for immunophenotyping and cytokine detection. For that purpose, PBMCs were incubated for 48 h with 2 µg/mL of F4 as specific stimulus. RPMI and 10 µg/mL of concanavalin A (Sigma-Aldrich, Madrid, Spain) were used as negative and positive controls for the assay, respectively. Supernatants were collected and stored at −80 °C for cytokine analysis. PBMCs were harvested and washed

Table 1
List of antibodies used for the detection of surface markers.

Marker	Host	Antibody isotype	Target	Clone	Fluorochrome	Brand	Dilution
CD3ε	Mouse	IgG2a	Pig	BB23-8E6-8C8	PE-Cy7	BD Pharmingen	1:200
CD4	Mouse	IgG2b	Pig	74-12-4	Alexa Fluor 647	BD Pharmingen	1:200
CD8a	Mouse	IgG2a	Pig	76-2-11	FITC	BD Pharmingen	1:200
CD27	Mouse	IgG1	Pig	B30C7	Stained with APC-Cy7 secondary antibody	BIO RAD	1:100 (sec 1:400)
CD154 (CD40L)	Mouse	IgG1	Human, Monkey	5c8	In-house conjugated with Mix n' Stain CF405L (Biotium, Fremont, CA, USA)	BIOxCELL	1:100

with FACS buffer (0.5% FBS-PBS). After washes, cells were surface stained for 45 min at room temperature with a mixture of labelled antibodies at the dilution indicated in Table 1 in FACS buffer. After staining, cells were washed again and analyzed in a MACSQuant 10 Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany), acquiring twenty-thousand events per sample to perform the analysis. Unstained cells, stained samples with viability marker (Live-or-Dye™ 405/545 Fixable Viability Staining Kit, Biotium, Fremont, CA, USA), isotypes for each antibody subclass, and Fluorescence Minus One (FMO) stained samples were included as controls to adjust the analysis and discard false positive results. Analysis of the results was carried out with FLOWLOGIC software v7.3 (Inivai Technologies, Melbourne, Australia). Dead cells were excluded from the analysis according to the pattern obtained by the fixable cell-viability staining mentioned above. The gating strategy can be referred in supplementary Figure S1.

Harvested supernatants from *in vitro* PBMC stimulation were used for IFNα, IFN-γ, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12/IL-23p40 and TNF-α determination using Invitrogen™ Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Immunoassay kit (Thermo Fisher, Madrid, Spain), following manufacturer's indications. Supernatants were analyzed using a MagPix® xMAP® Analyzer (Luminex Corporation, Austin, TX, USA). Cytokine concentrations were determined using the xPONENT® software. TGF-β detection was individually evaluated in same supernatants by using the TGF-β Human Matched Antibody Pair kit from Invitrogen (Carlsbad, CA, USA), following manufacturer's instructions.

Statistical analysis and modelling

Before conducting any statistical analysis, data were screened for unlikely, extreme, or missing values and no data were excluded based on these premises. First of all, a descriptive analysis was carried out with the main variables of interest: experimental groups (F4+CAF®01, F4+CDA, PBS and Porcilis® Glässer), colonization with virulent *G. parasuis* at D38 (yes or no), sow origin (n = 8 sows), sow parity (3, 4 and 5), total anti-*G. parasuis* antibody ratio (doubtful, positive and negative values by the Ingezim-Haemophilus ELISA kit), and anti-F4 IgG levels found in sows by the above described F4 ELISA. Different statistical models of linear regression were additionally run for the following continuous outcome variables: IgG F4 D38/anti-F4 IgG levels in sow, ELISPOT Nagasaki D21, proportion of T helper (CD4+) F4, T-memory (CD4+CD8+), Central T-memory (CD4+CD8+CD27+) and Effector T-memory T (CD4+CD8+CD27-) cells, and IFN-α and TGF-β levels found in supernatants after 48 h of stimulation with the F4 fragment. The outcome variables that showed a skew pattern were, therefore, transformed by taking their natural logarithm or log10 and a univariable analysis was done to test the unconditional associations between outcome and different explanatory variables of interest. At this initial screening, explanatory variables with $p < 0.25$ were included in multivariable linear regression models according to Dohoo et al. [29].

The significant independent variables from the univariable analysis were then offered to a multivariable mode and a manual backward elimination was implemented, to obtain a final model that exclusively included variables with a p value < 0.05, considered as significant. The p value and the regression coefficient (b) with a 95% confidence interval (95% CI) were reported for each variable. All these statistical analyses were conducted using the R version 3.3.3 software (R Core Team, 2015).

Significance of the proportions of colonized animals among experimental groups was calculated with the Comparison of proportions calculator v20.206. https://www.medcalc.org/calc/comparison_of_proportions.php (MedCalc Software Ltd, Ostend, Belgium), which uses the "N-1" Chi-squared test as recommended by Campbell [30] and Richardson [31]. The confidence interval was calculated according to Altman et al. [32]. Graphs were plotted using Prism v9 (GraphPad Software, San Diego, CA, USA).

Results

Natural colonization by a virulent G. parasuis strain was modulated by F4-vaccination

A natural nasal colonization by virulent serovar 4 *G. parasuis* was detected by PCR at D0 (before immunization started) in four unvaccinated control animals (Fig. 2). Further PCR analyses evidenced that the colonization spread to the rest of the piglets and pens, as was observed on day 21, when nasal colonization was common in all the groups (6/7 [86%] in PBS, 7/7 [100%] in F4+CAF®01, 5/7 [71%] in F4+CDA and 1/2 [50%] in Porcilis® Glässer). Interestingly, at day 38 (17 days after vaccine boosting), the colonization by serovar 4 virulent *G. parasuis* was significatively reduced in the F4+CAF®01 immunized pigs ($p = 0.02$), with a 71% of reduction, from 100% (7/7) at D21 to 28.5% (2/7) at D38 (Fig. 2). The reduction of the virulent/serovar 4 *G. parasuis* presence was not that evident in the rest of the groups, with a reduction of 16.6% in control non-immunized pigs PBS-NV (from 86% (6/7) to 71% (5/7)); and a 20% reduction for the F4+CDA vaccinated piglets (from 71% (5/7) to 57% (4/7)), respectively (Fig. 2).

F4+CDA induced significantly higher anti-F4 specific IgG1 antibodies than F4+CAF®01

The kinetics of anti-F4 specific IgG induction were evaluated by ELISA using F4-coated plates and sera from all animals prior to immunization (D0), after the first immunization (D21) and after boosting (D38). As shown in Fig. 3A, at D38, animals vaccinated with F4+CDA showed significantly higher antibody levels than the PBS-NV control pigs ($p < 0.01$). Similar results were found for the anti-F4 IgG1 immunoglobulin isotype (Fig. 3B), but not for the anti-F4 specific IgG2 (Fig. 3C) and IgM (Fig. 3D). Individual kinetics of specific anti-F4 IgGs can be found in supplementary Figure S2.

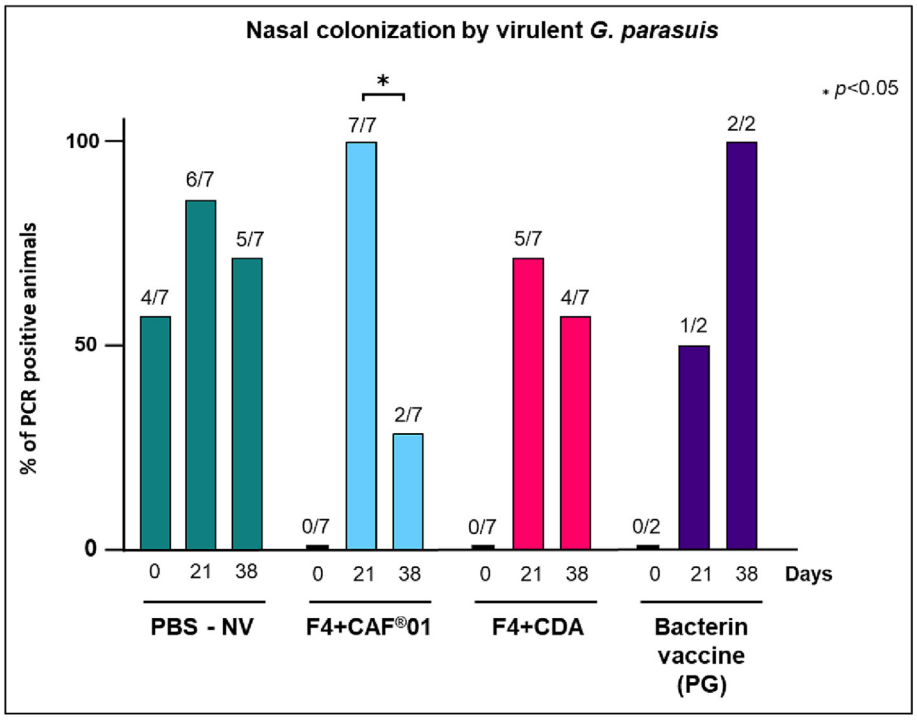


Fig. 2. Nasal colonization of the piglets by virulent G. parasuis during the vaccination experiment detected by LS-PCR from nasal swabs collected at 0-, 21- and 38-days post-vaccination. Bars represent the percentage of positive animals to virulent strains within the group in the different timepoints. Above each bar is depicted the number of positive animals out of the total. Positive signals detected by LS-PCR were later confirmed to belong to serovar 4 by G. parasuis serotyping PCR.

Before immunization (D0), the F4-ELISA also detected low levels of anti-F4 IgGs (Fig. 3A), mostly of the IgG2 isotype (Fig. 3C), which reduced their presence by D21 in all animals, indicating their maternal origin. Except for the pigs immunized with F4+CAF®01, the overall levels of F4 anti-IgG2 antibodies tended to increase after D21 (Fig. 3C), most probably reflecting the natural colonization with the *G. parasuis* strain (serovar 4) together with the vaccination in those vaccinated groups. Variable values of total anti – *G. parasuis* were found at 0 DPV in all the groups. Only the two animals immunized with the bacterin vaccine Porcilis® Glässer showed a rise of total anti – *G. parasuis* antibodies on 38 DPV (supplementary Figure S3).

Vaccination and colonization affected T-cell specific F4 responses

Specific secretion of IFN-γ by F4-stimulated PBMCs was already detected by ELISPOT in piglets before vaccination (D0), a fact that might indicate early colonization by *G. parasuis* and/or maternal transfer of F4-specific T-cells to their offspring. Conversely to what was found for maternal anti-F4 antibodies, the number of F4-IFN-γ specific spots notably increased in all groups by D21, independently of the treatment received and coinciding with the peak of virulent *G. parasuis* detection in their nasal cavities (Fig. 4). After the second vaccination, the levels of F4-specific IFN-γ secretion proportionally decreased in all animals, including those in the control group. The individual kinetics of IFN-γ secreting cells are available in supplementary Figure S4. In fact, colonization status at D38 was significantly associated with the IFN-γ results in the ELISPOT at D21, using bacterin as stimulus (supplementary Figure S5); i.e., the piglets with higher IFN-γ response against the whole bacterium at D21, had higher probability to harbor virulent *G. parasuis* in their nasal cavities at D38 (22.00 [-17.95–61.95], p = 0.0021).

A closer look at the individual responses for anti-F4 IgG and IFN-γ secretion revealed that even though the strongest values of specific antibodies were found in the pigs vaccinated with F4 +CDA, these results did not completely correlate with the number of IFN-γ secreting cells (Fig. 5). Interestingly, two piglets in the CDA group did not get colonized at any time of the study and showed increase in both IFN-γ and IgG responses comparing D38 and D21, while a third piglet cleared the colonization by D38 and also showed an increase in IFN-γ and IgG responses. On the other hand, the net increase of F4 specific antibodies in some piglets of the unvaccinated group indicates that the presence of F4-specific anti-IgG or IFN-γ secreting cells are potential indicators of colonization by virulent *G. parasuis* (Fig. 5).

For the analysis of the cell-mediated immune response at D38, the experimental groups were split according to their nasal colonization status on this timepoint. Evaluation of PBMCs after *in vitro* restimulation with F4 revealed some trends in T helper cells (CD3+CD4+), memory T cells (CD3+CD4+CD8+), effector memory T cells (CD3+CD4+CD8+CD27-) and central memory T cells (CD3 +CD4+CD8+CD27+) in the pigs immunized with the F4 combinations (Fig. 6). Thus, the animals vaccinated with F4+CAF®01 showed a reduction of the percentage of all of the above cell subsets in the colonized animals with respect to the uncolonized ones. Colonization status did not influence the proportion of those cell subsets in the F4+CDA immunized pigs. On the other hand, the non-vaccinated control group showed higher proportions of the cell subsets in colonized piglets (Fig. 6). CD154 signal was not detected in any of the samples.

Albeit not statistically significant, the amount of TGF-beta and IL-8 found in the supernatant of PBMCS after F4-*in vitro* stimulation was higher for the F4+CAF®01 pigs than for the rest of the groups (Fig. 7).

Maternal immunity and piglets' colonization affected the immunological outcome

The multivariable statistical model performed to evaluate possible relations among variables showed a significant association between the amount of IFN-γ secreting cells from the piglets and

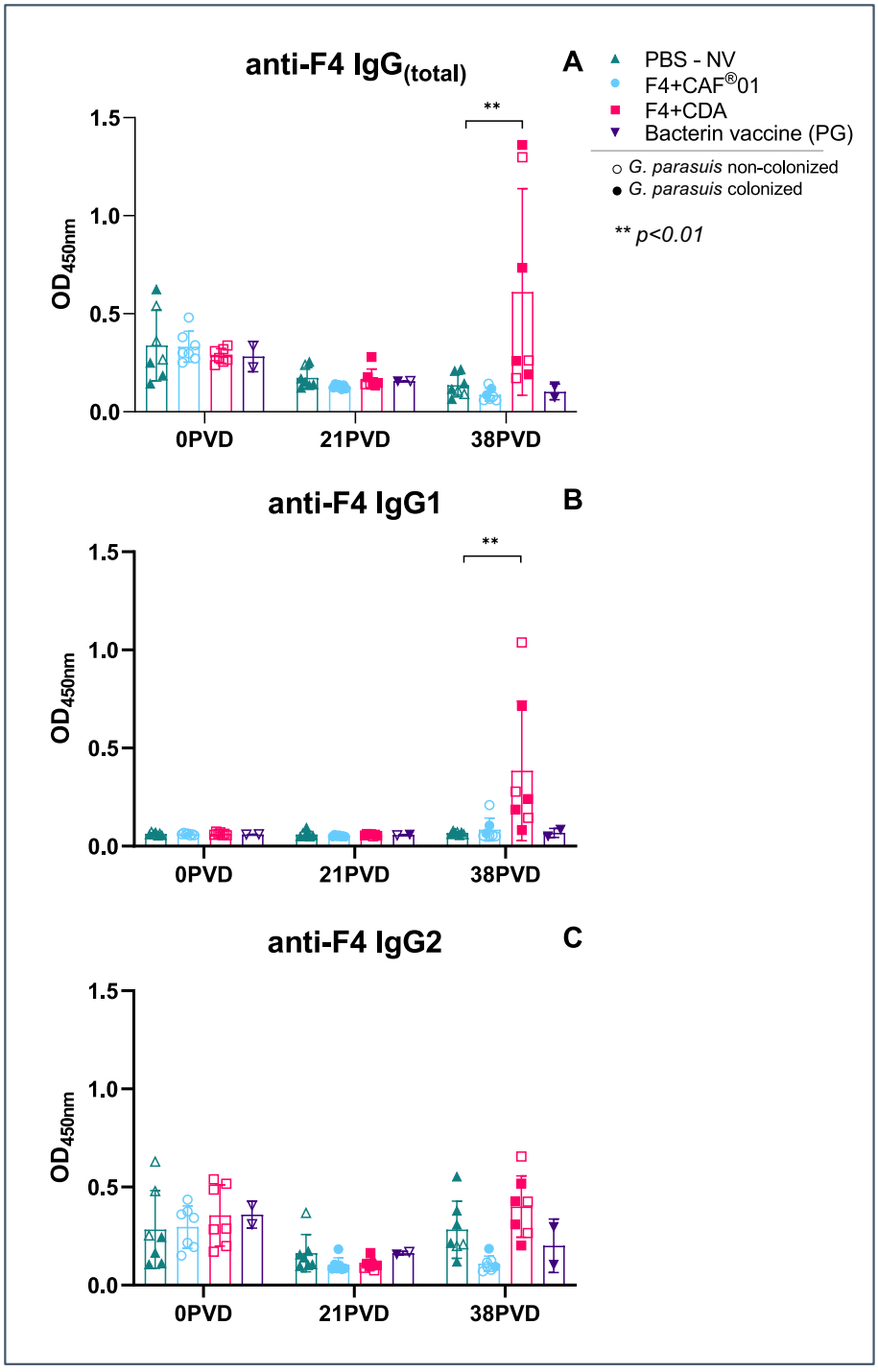


Fig. 3. F4-specific antibodies detected in sera from pigs immunized with either F4+CDA, F4+CAF®01, PBS or Porcilis® Glässer along the experimental procedure from days 0-, 21- and 38-days post-vaccination. Anti-F4 specific antibody kinetics: Total IgG (A), IgG1 (B) and IgG2 (C). ODs obtained for each pig are plotted with symbols, averages and standard are also plotted. Individual piglets colonized at each time point by virulent G. parasuis are represented with filled symbols, whereas non-colonized animals are represented with empty symbols. Statistical significance (*) and p values are indicated accordingly.

their sow of origin. According to the results, the sow origin affected the IFN-γ secreting PBMC in the piglets with a *p* value of 1.36e-08, and specifically the sow's anti-F4 IgGs were positively associated with the IFN-γ secreting cells of the offspring before boosting (D21) with a *p* value of 0.02556. Moreover, the final model showed a significant positive association between the secreted IFN-γ on D21, and the colonization by virulent *G. parasuis* detected 17 days later with a *p* value of 0.0021.

The sow parity negatively affected the expression of memory F4 specific Th helper cells ($p = 0.02$), central memory T cells ($p = 0.02$) and effector memory T cells ($p = 0.04$). Similarly, TGF-β secretion by piglets' stimulated PBMCs was positively associated with the

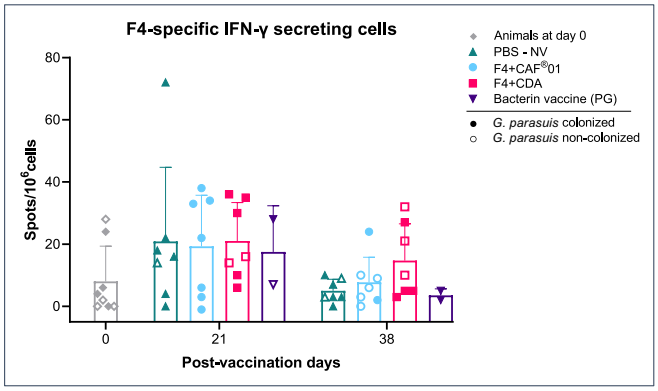


Fig. 4. F4-specific IFN-γ secreting cells measured by ELISPOT. PBMCs ($5x10^5$) collected at different times during the study were plated and stimulated for 48 h with 2 μg/mL of F4. Individual piglets colonized at each timepoint by virulent G. parasuis are represented with filled symbols, whereas non-colonized animals are represented with empty symbols. Averages and standard deviations found in each group are also plotted. On day 0, 8 piglets (that later belonged to PBS-NV and Porcilis® Glässer groups) are represented in the same bar, as all piglets were naïve at this timepoint.

anti-F4 specific antibodies found in the respective sow of origin ($p < 0.05$).

Discussion

The immunity elicited by both F4-adjuvanted formulations presented in this study was highly variable within groups, and we identified parameters of maternal influence together with the nasal colonization with virulent *G. parasuis* as the sources of this high individual variability. This latter fact had a higher impact on the analyzed immune parameters, as observed in the non-vaccinated group. Regardless of these facts, the experimental vaccine formulations were capable to trigger different immune responses in the piglets, an important circumstance considering the young age of the animals and the bacterial colonization, fact that occurs often under natural conditions [1].

The piglets immunized with the CDA combination elicited an efficient induction of anti-F4 IgG1 antibodies by D38 (17 days after boosting), indicating a Th2 skewed response. In addition, two F4 +CDA vaccinated animals did not become colonized during the experiment, displaying a congruent humoral and cellular immune response. Similar humoral systemic responses were previously reported with other antigens from diverse pathogens adjuvanted with STING agonists (CDA or cyclic di-GMP) in mice [33,34]. However, in our case, the delivery system of the vaccine formulation might also have affected the development of a proper mucosal response. A different delivery system of administration may have been more efficient in terms of both humoral and cell-mediated immune responses, as reported in pigs intramuscularly vaccinated with a *Mycoplasma hyopneumoniae* bacterin adjuvanted with liposome-encapsulated CDA [35]. At this point, it is important to note that cyclic dinucleotide adjuvants, like CDA, stimulate efficient mucosal responses when administered by either nasal [36,37] or oral routes [38] rather than intramuscularly. This might be instrumental to achieve both efficient protection against infection and reduction of horizontal transmission. According to our results, F4-specific systemic antibodies might not correlate with a putative protection against natural colonization by virulent *G.*

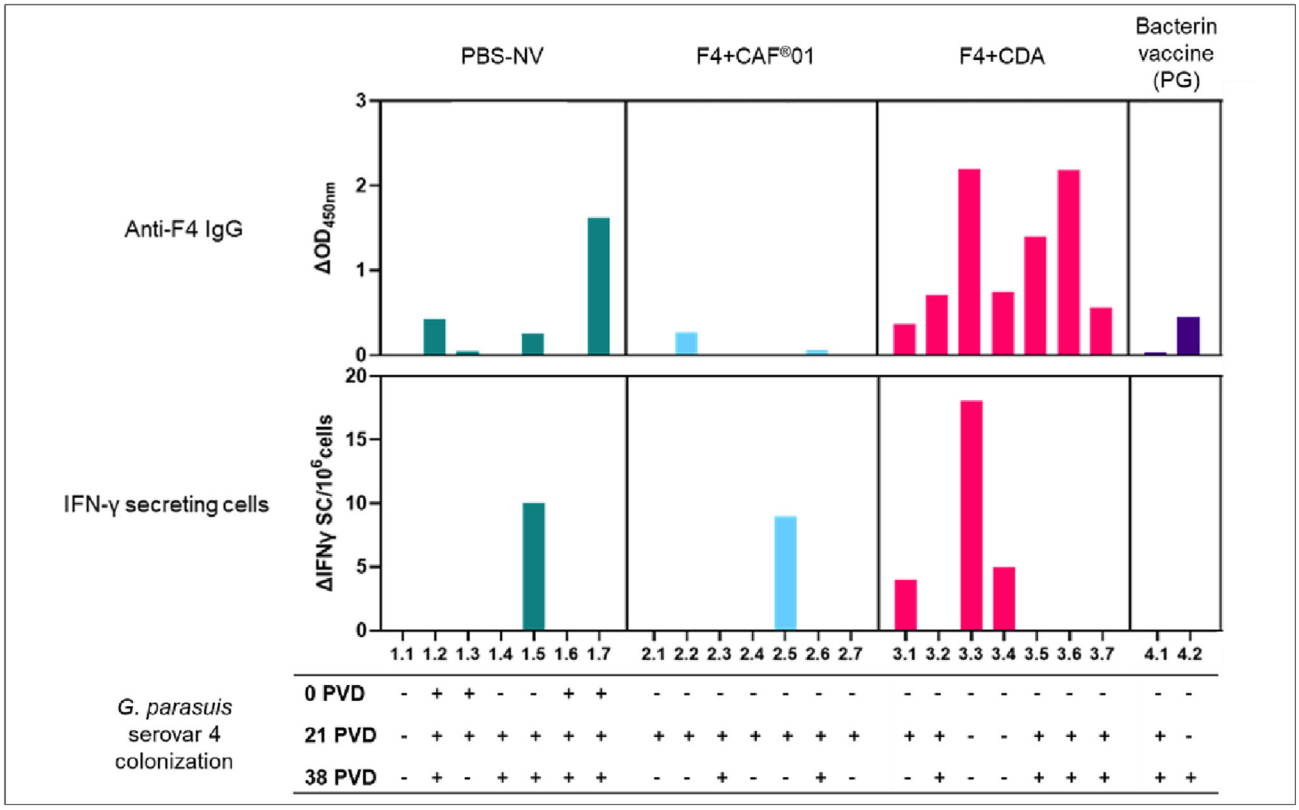


Fig. 5. F4-specific immune responses, antibodies (above) and IFN-γ secreting T-cells (below) found in individual pigs injected with either PBS, F4+CAF®01, F4+CDA, or Porcilis® Glässer. Δanti-F4 IgG result from subtracting the values obtained on day 21 (post-priming) to the results of day 38 (post-boost). ΔIFN-γ-SC is the result of the subtraction of the spots obtained after boost D38 to the results after priming D21. Nasal colonization by G. parasuis serovar 4 is indicated at the bottom of the figure.

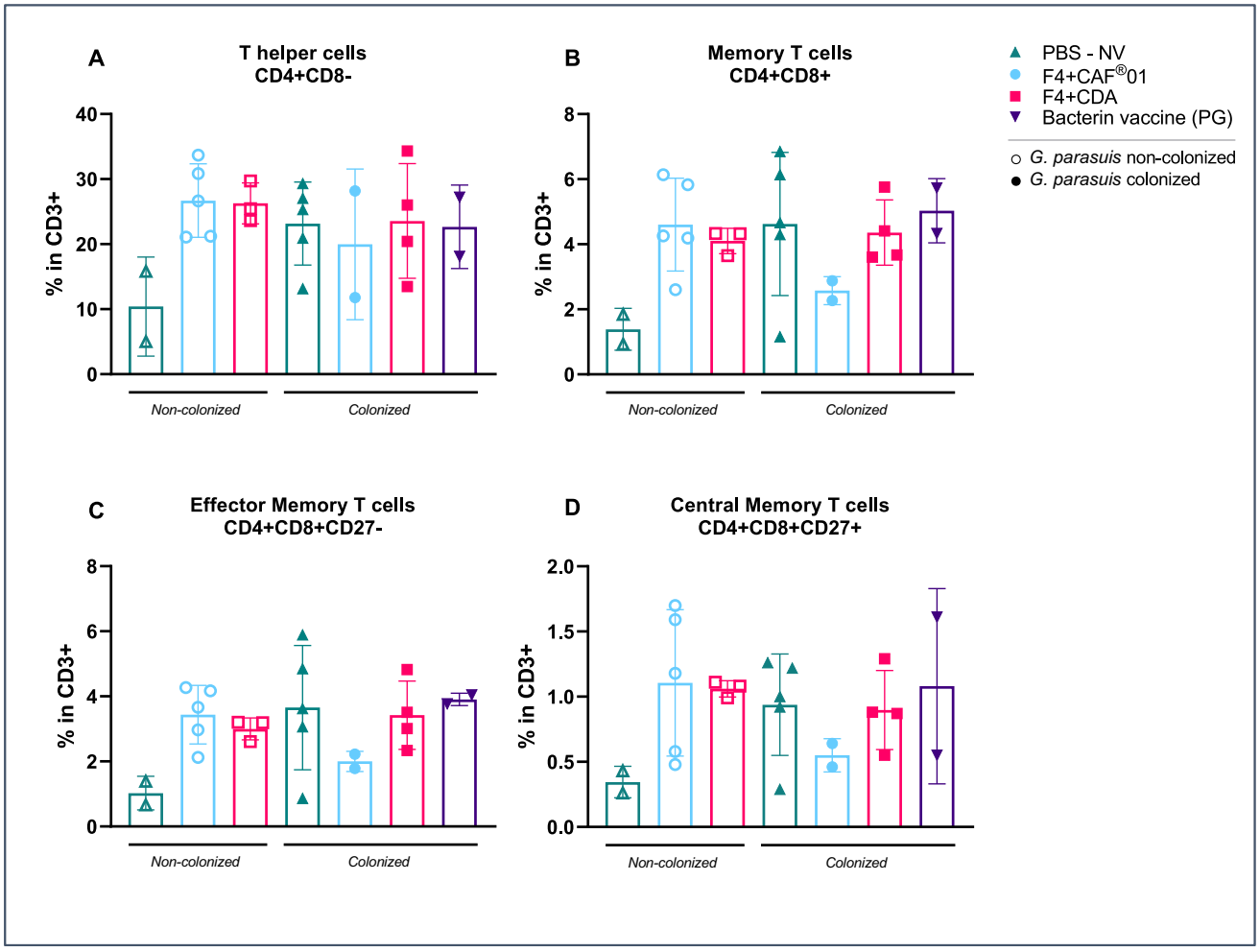


Fig. 6. Proportion of T cell populations found on *in vitro* F4-stimulated PBMCs collected on day 38 and analyzed by flow cytometry. Results are expressed after gating of twenty thousand events acquired on total PBMCs. Individual piglets colonized at this timepoint by virulent G. parasuis are represented with filled symbols, whereas non-colonized animals are expressed with empty symbols. Averages and standard deviations found in each group are also plotted.

parasuis, in agreement to the results observed with antigens from similar bacteria [39–41].

The piglets vaccinated with F4+CAF®01, on the contrary, did not display a significant humoral response against the immunogen. Only two animals showed a low F4 antibody signal that might be attributed to the colonization by *G. parasuis*. Generalized lack of antibody response can be explained by the interference of maternal antibodies in the maturation of B cells helped by the follicular T cells (Tfh) in the germinal centers of the lymph nodes as suggested by Vono et al. [42]. On the other hand, one can also think that the early T cell priming redirects to mucosal tissues and the systemic immunity in this case was poorly induced [43]. In agreement with this idea and previous observations with CAF®01, F4+CAF®01 vaccination significatively reduced the nasal colonization by *G. parasuis* [28,44,45]. A reduction of *G. parasuis* nasal colonization after vaccination has been also shown with another subunit vaccine, TbpB, using different adjuvants and administration routes [46]. The clearance observed in our study might indicate a primed mucosal response with a mechanism still to be determined as a result of the adjuvant effect characterized by a Th17-type response elicited by both adjuvants.

In previous neonatal studies performed in mice, CAF®01 and CDA adjuvants proved a complete efficacy and immunogenicity [21,25]. However, the animals used in the mentioned studies belonged to naïve mothers and, therefore, a proper maturation of the germinal centers was possible leading to an efficient humoral response without interaction with maternally-derived antibodies. According to our results, CDA seems to help overcoming the presence of maternally derived antibodies, at least partially, suggesting an alternative way of immune stimulation. From this perspective, previous studies in mice attributed the enhanced antibody response to an autonomous activation of B cells through the STING signaling pathway [47].

In the case of the CAF®01 formulation, we also cannot rule out the effect of the antigen adsorption into the liposome formulation. Indeed, a recent study demonstrated that changes of the electrostatic interactions between antigens and CAF®01 can lead to different immune responses [48]. Thus, the authors associated efficient Th1/Th17-type responses to higher adsorption rates of the antigen into the vaccine formulation, in inverse correlation with antibody titers. Of note, the generation of opsonizing antibodies is considered crucial for protection against Glässer's disease [49,50] and maternally-derived immunity plays an important role protecting the offspring by passive transfer via colostrum during their first weeks of life [51].

Despite the limitations of this study, the situation offered the opportunity to evaluate the effectivity of the different vaccine combinations under a natural exposure to the pathogen, under conditions closer to a conventional farm. Since a relatively low systemic humoral response as observed after F4+CAF®01 immunization did not correlate with the protective efficacy, the putative mucosal response may be useful against the colonization of patho-

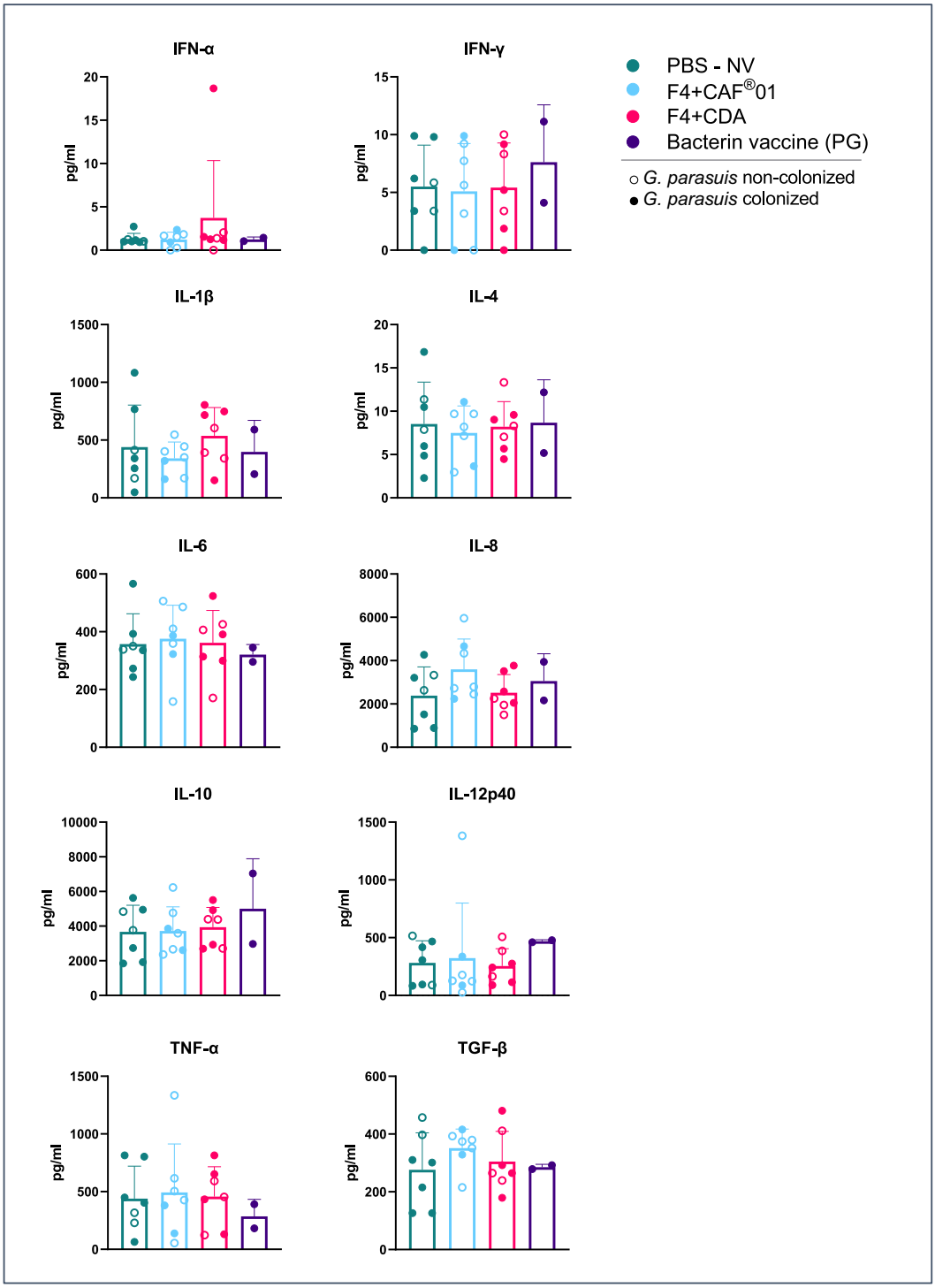


Fig. 7. Cytokine profiles obtained by ProcartaPlex™ immunoassay and TGF-β ELISA from the supernatants of F4- stimulated PBMCs on day 38 from all pigs. Individual pigs colonized on this time point by virulent G. parasuis are represented with filled symbols, while non-colonized animals are expressed with empty symbols. Averages in bars and standard deviations found in each group are also plotted.

genic respiratory bacteria. Therefore, it would be of interest to address in future studies the mucosal humoral and cell-mediated response in the upper respiratory tract of the piglets after vaccination with CAF®01 or CDA adjuvanted antigens.

The multivariable model indicated correlation between sow parity and amount of T helper and memory T cells in the piglets, as reported after a cell-subset assessment in sows of diverse parities [52]. Likewise, the model showed an association between secreted TGF-β by the *in vitro* stimulated PBMCs and the level of specific anti-F4 IgGs in the sow of origin. This association of the TGF-β levels together with an increase anti-F4 antibodies are in line with the ones published in a previous study of maternal vaccination, where TGF-β in sera and specific antibodies against F4 antigen were upregulated in the offspring of F4-vaccinated sows [16]. This feature, together with a slight increase of Th17-like response cytokines, might indicate an intrinsic immunogenicity of the vac-

cine protein that requires further attention. Although not yet described, it cannot be ruled out that the processing and presentation of F4-protein might result in a microenvironment favored towards a Treg or Th17 polarization, since cytokines related to these responses were described recently for *G. parasuis* [53].

In summary, the immune responses observed in vaccinated and non-vaccinated piglets were affected by the adjuvant included in the vaccine but also by the sow and the natural colonization by a virulent strain of *G. parasuis*. The F4 conserved fragment currently represents a good marker for the detection of colonization/infection, either by PCR detecting the F4 gene or measuring specific F4 antibodies, making the F4 conserved fragment not only a vaccine candidate but also an indicator to include for diagnostics of immunity against *G. parasuis* in pigs. Further studies should attempt to identify the delicate balance required to ensure protection against virulent *G. parasuis,* including evidence of the responses induced in lymphoid and mucosal tissues.

Patents

Carlos A. Guzmán and Thomas Ebensen are named as inventors in a patent covering the use of CDA as a neonatal adjuvant (EP 19193982), which was previously patented covering the use of CDA as adjuvant (PCT/EP 2006010693).

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CRediT authorship contribution statement

Sergi Lopez-Serrano: Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Yasser S. Mahmmod:** Software, Formal analysis. **Dennis Christensen:** Conceptualization, Resources, Writing – review & editing. **Thomas Ebensen:** Conceptualization, Resources, Writing – review & editing. **Carlos A. Guzman:** Conceptualization, Resources, Writing – review & editing. **Fernando Rodríguez:** Writing – review & editing. **Joaquim Segalés:** Project administration, Conceptualization, Funding acquisition. **Virginia Aragón:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2023.100330.

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