Universitat Autònoma de Barcelona

Immune response to vaccination with CAF®01 or CDA adjuvanted conserved antigens from porcine viral and bacterial pathogens

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Tesi doctoral presentada per Sergi López Serrano per accedir al títol de Doctor en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autónoma de Barcelona. Aquesta tesi ha estat dirigida per la Dra. Virginia Aragón Fernández, el Dr. Ayub Ismail Darji i el Dr. Joaquim Segalés Coma.

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La Dra. Virginia Aragón i el Dr. Ayub Ismail Darji, investigadors de l'Institut de Recerca i Tecnología Agroalimentàries – Centre de Recerca en Sanitat Animal (IRTA – CReSA) i el Dr. Joaquim Segalés, catedràtic del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit a l'IRTA-CReSA,

Certifiquen:

Que la memòria titulada "**Immune response to vaccination with CAF®01 or CDA adjuvanted conserved antigens from porcine viral and bacterial pathogens**" presentada per Sergi López Serrano per l'obtenció del títol de Doctor en Medicina i Sanitat Animal s'ha realitzat sota la seva supervisió i tutoría, i n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 21 de Juny de 2022.

Dra. Virginia Aragón Fernández	Dr. Ayub Ismail Darji	Dr. Joaquim Segalés Coma	Sergi López Serrano
Directora	**Director**	**Director i Tutor**	**Doctorant**

A tu, per ser sempre al meu costat incondicionalment…

Si no conozco una cosa, la investigaré.

Louis Pasteur

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Index

Abbreviations

APCs: Antigen-presenting cells

BCR: B-cell receptor

C05: *S. suis* antigen SSU0185

CAF: Cationic Liposome Formulation

CD: Cluster of differentiation

CDA: c-di-amp or bis-(3′,5′)-cyclic dimeric adenosine monophosphate

CFA: Complete Freund's Adjuvant

CPS: Capsular polysaccharide

CTL: Cytotoxic T cells

DAMPs: Danger/damage-associated molecular patterns

DCs: Dendritic cells

DDA: N,N-dimethyl-N,N-dioctadecylammonium

EF: Extracellular factor

FOXP3: Fork-head box p3

GATA3: GATA binding protein 3

HA: Hemagglutinin

HI: Hemagglutination inhibition

HIA: Hemagglutination inhibition assay

HPAIV: Highly Pathogenic Avian Influenza Virus

IFA Incomplete Freund's Adjuvant

IFN-α: Interferon-alpha

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL-10: Interleukin 10

IL-12 p40: Interleukin 12 p40

IL-1β: Interleukin 1 beta

IL-2: Interleukin 2

IL-4: Interleukin 4

IL-6: Interleukin 6

IL-8: Interleukin 8 (CXCL8)

iNKT: Invariant natural killer T cells

IV: Influenza virus

LPS: Lipopolysaccharide

MHC: Major histocompatibility complex

Mincle: Macrophage inducible Ca^{2+} dependent lectin receptor

MRP: Muramidase-released protein

MRP2: Muramidase-released protein from serovar 2

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK cells: Natural killer cells

NKT: cells Natural killer T-cells

NOD-like receptors: Nucleotide-binding oligomerization domain-like receptors

O/W: Oil-in-water emulsion

PAMPs: Pathogen-associated molecular patterns

PRRs: Pattern recognition receptors

RORγT: Retinoic acid receptor-related orphan receptor gamma T

STING: Stimulator of Interferon genes

Tbet: T-box expressed in T cells Transcription factor

TCR: T-cell receptor

TDB: α,α-trehalose 6,6′-dibehenate

TGF-β: Transforming growth factor-beta

Th: T helper

TLRs: Toll-like receptors

TNF-α: Tumoral necrosis factor Alpha

Treg: T regulatory response

VtaA: Virulent-trimeric autotransporters A

W/O: Water-in-oil emulsion

W/O/W: Water-in-oil-in-water emulsion

WHO: World Health Organization

WOAH: World Organisation for Animal Health

αGC: α-Galactosyl ceramide

αGCM: αGalCerMPEG α-Galactosyl ceramide multi polyethylene glycol

Abstract

The swine is especially susceptible to a wide variety of pathogens at younger ages as for instance viruses like influenza virus (IV), or bacteria such as *Glaesserella parasuis*, and *Streptococcus suis*. Disease caused by these pathogens can be prevented by good husbandry practices with the herds or by antimicrobial treatment, and to a lesser extent, vaccination. However, due to the risk of new emerging strains and antimicrobial resistances compels to look for effective alternatives to control infections and improve the animal health. In this respect, vaccination is probably the best tool for infectious diseases prevention. Nonetheless, current vaccines do not confer long lasting or protective immune responses against heterologous strains of these pathogens. For this reason, the use of relatively well-conserved antigens may become an approach to obtain immunogenic, effective, and safe vaccines.

In this PhD dissertation, we aimed to identify effective antigen/adjuvant combinations capable of strengthening the innate and adaptive immune responses and confer protection against a given pathogen. Hence, the immunogenicity and efficacy of conserved antigens were assessed in combination with the liposome-based adjuvant CAF®01 or the cyclic dinucleotide CDA.

The first study of this work (chapter III) assessed the immunogenicity and efficacy of IV hemagglutinin peptide NG34 adjuvanted with CAF®01 or CDA/αGCM. Young pigs were twice immunized in an interval of 21 days and challenged afterwards with pdmH1N1 strain by the intranasal and endotracheal routes. In this experiment,

NG34+CAF®01 immunized animals elicited a stronger specific humoral and cell mediated immune response with high amounts of specific IgGs and IFN-γ secreting cells that correlated with less pathology and viral load in lung. On the contrary, NG34+CDA/αGCM formulation showed a weak immune response correlated with variable IV compatible lesions and no reduction of viral load.

In the second study (chapters IV and V), the vaccination with the F4 protein (conserved protein fragment of virulent strains of *G. parasuis*) was evaluated in neonate piglets. The study is divided in two parts: in the first part, the immune responses to neonate vaccination with CAF®01 or CDA adjuvanted F4 protein was assessed; in the second part, results of the intraperitoneal challenge with serovar 5 of *G. parasuis* are presented. Soon after the beginning of the study a natural nasal colonization by a heterologous strain of *G. parasuis* was detected in the animals. Piglets vaccinated with F4+CAF®01 were capable of reducing the bacterial colonization in their nasal cavities on day 38 in the absence of specific antibodies. It was also observed an enhanced, albeit variable specific humoral response in animals immunized with F4+CDA. Similarly, cell-mediated immune responses were evaluated by flow-cytometry where it was noted that nasal colonization by the heterologous strain of *G. parasuis* affected the immune parameters far beyond than expected. In the second part of the study (chapter V), the intraperitoneal inoculation with serovar 5 of *G. parasuis* is described. Challenge affected severely all the groups without differences, hampering the correct evaluation of the vaccine candidate's efficacy.

Finally, the third study (chapter VI) evaluated the immunogenicity and efficacy of two cell-wall proteins of *Streptococcus suis* (MRP2 and C05 antigens). New-born piglets were twice vaccinated with both antigens adjuvanted with either CAF®01 or CDA and finally challenged by intranasal inoculation with serovar 2 of *S. suis*. In this experiment both combinations elicited weak immune responses, unable to protect the animals against the infection unlike piglets immunized with autogenous vaccine.

Resum

El porc és susceptible a una gran varietat de patògens en edats primerenques com per exemple el virus d'influença (VI) o els bacteris *Glaesserella parasuis* i *Streptococcus suis*. Avui dia els efectes d'aquests patògens poden evitar-se amb una bona gestió sanitària dels ramats o amb tractament antibiòtic, i en menor mesura, a través de vacunació. No obstant, tant l'emergència de noves variants víriques així com també de noves soques bacterianes amb resistències antimicrobianes fa necessari buscar alternatives efectives per combatre les infeccions i així millorin la salut dels animals. En aquest sentit, la vacunació representa una excel·lent estratègia per prevenir les malalties infeccioses. Tanmateix, les vacunes disponibles contra aquestes malalties no ofereixen una resposta immune prolongada o protectora contra diferents soques del mateix patogen. Per això, l'ús d'antígens relativament conservats pot esdevenir una estratègia per aconseguir vacunes immunogèniques, efectives i segures.

En aquesta Tesi doctoral es proposa identificar una combinació efectiva d'antigen/adjuvant capaç de reforçar la resposta immunitària innata i adaptativa i així conferir protecció contra un determinat patogen. Per a això s'han avaluat en tres estudis la immunogenicitat i eficàcia d' antígens conservats en combinació amb l' adjuvant liposòmic CAF®01 o el dinucleòtid cíclic CDA.

El primer estudi (capítol III), avalua l'eficàcia i immunogenicitat del pèptid de l'hemaglutinina NG34 adjuvantat amb CAF®01 o amb la combinació de CDA/αGCM. Garrins joves es van vacunar dos cops en

un interval de 21 dies i infectar experimentalment amb una soca de VI pandèmica pdmH1N1 per vies intranasal i endotraqueal. La vacunació amb NG34+CAF®01 va produir mes anticossos específics i cèl·lules secretores d'IFN-γ, que a més es va correlacionar amb una menor patologia en pulmó així com també menor carrega vírica. En canvi, la formulació NG34+CDA/αGCM va generar una resposta immune dèbil sense reducció de càrrega vírica i amb una variable patologia compatible amb Influença.

En el segon estudi (capítols IV i V) s'analitza la vacunació neonatal amb el fragment proteic F4, una seqüència conservada en les soques virulentes de *G. parasuis*. L'estudi es divideix en dues parts: en la primera part es descriu la resposta immune després de la vacunació de amb la proteïna F4 adjuvantada amb CAF®01 o amb CDA mentre que en la segona part es detalla el procés d'infecció dels animals després d'inocular el serotip 5 de *G. parasuis* per via intraperitoneal. A l'inici de l'estudi es va detectar una colonització nasal natural per part d'una soca heteròloga de *G. parasuis*. Els animals vacunats amb la combinació d' F4+CAF®01 van ser capaços de reduir la càrrega d'aquesta soca a dia 38 post-vacunació en absència d'anticossos. També es va observar que la combinació d'F4+CDA va generar una resposta humoral específica amb IgGs anti-F4 encara que variable dins del grup. Així mateix es van valorar la resposta immune cel·lular mitjançant citometria de flux, on es va veure que la colonització amb *G. parasuis* va afectar els paràmetres analitzats. En la segona part de l'estudi, es descriuen els efectes de la inoculació intraperitoneal en els animals vacunats, on es va poder

copsar l'afectació generalitzada en tots els grups sense diferències, dificultant la correcta avaluació dels candidats vacunals.

Finalment, en el tercer estudi (capítol VI) s'examina la immunogenicitat i eficàcia de dues proteïnes de la paret cel·lular d'*S. suis*: els antígens MRP2 i C05. Garrins nounats es van vacunar en dues tandes amb ambdós antígens adjuvantats amb CAF®01 o bé amb CDA i infectats experimentalment intranasalment amb una soca de serotip 2 d'*S. suis*. Ambdues combinacions van produir una immunogenicitat dèbil, tant humoral com cel·lular, incapaç de protegir els animals front a la infecció a diferencia dels animals immunitzats amb autovacuna.

Resumen

El cerdo es una especie susceptible a una gran variedad de patógenos que afectan a su salud especialmente en edades tempranas como por ejemplo el virus de influenza (VI), o las bacterias *Glaesserella parasuis* y *Streptococcus suis*. Actualmente la infección por dichos patógenos puede evitarse gracias a una buena gestión sanitaria de los rebaños o con tratamiento antibiótico y, en menor medida, a través de vacunación. Sin embargo, la emergencia de nuevas variantes víricas o cepas bacterianas con resistencias antimicrobianas obliga a buscar alternativas efectivas para combatir las infecciones y así mejorar la salud de los animales. En este sentido, la vacunación representa una excelente herramienta para prevenir las enfermedades infecciosas. Sin embargo, las vacunas disponibles frente a las enfermedades mencionadas no ofrecen una respuesta inmune prolongada o protectora contra diferentes cepas del mismo patógeno. Por esta razón, el uso de antígenos relativamente conservados puede convertirse en una estrategia para obtener vacunas inmunogénicas, efectivas y seguras.

En la presente Tesis Doctoral se propone identificar una combinación efectiva de antígeno/adyuvante capaz de reforzar la respuesta inmunitaria innata y adaptativa para así conferir protección contra un determinado patógeno. Para ello se han evaluado la inmunogenicidad y eficacia de antígenos conservados en combinación con el adyuvante liposómico CAF®01 o el dinucleótido cíclico CDA.

El primer estudio (capítulo III) evalúa la eficacia e inmunogenicidad del péptido NG34 de la hemaglutinina del virus de Influenza en

combinación con los adyuvantes CAF®01 o CDA/αGCM. Cerdos jóvenes fueron doblemente inmunizados y posteriormente infectados con una cepa de VI pandémica pdmH1N1 por vías intranasal y endotraqueal. La vacunación con NG34+CAF®01 produjo una mayor cantidad anticuerpos específicos y células secretoras de IFN-γ, que además se correlacionó con una menor patología en pulmón, así como menor carga vírica. Por el contrario, la formulación NG34+CDA/αGCM generó una respuesta inmune débil sin reducción de carga vírica y con una patología compatible con Influenza variable.

En el segundo estudio (capítulos IV y V), se analiza la vacunación neonatal con el fragmento proteico F4, una secuencia conservada en las cepas virulentas de *G. parasuis*. El estudio se divide en dos partes: en la primera parte se describe la respuesta inmune tras la vacunación con la proteína F4 en combinación con los adyuvantes CAF®01 o CDA, mientras que en la segunda parte se detalla el proceso de infección de los animales después de la inoculación intraperitoneal del serotipo 5 de *G. parasuis*. Al inicio del estudio se detectó una colonización nasal natural por parte de una cepa heteróloga de *G. parasuis*. Los animales vacunados con la combinación de F4+CAF®01 fueron capaces de reducir la carga de esta cepa a día 38 en ausencia de anticuerpos. También se observó que la combinación de F4+CDA generó una respuesta humoral específica, aunque variable dentro del mismo grupo. Asimismo, se valoró la respuesta inmune celular mediante citometría de flujo, donde la colonización con la cepa oportunista afectó a los parámetros analizados. En la segunda parte del estudio, se pudo apreciar que la infección intraperitoneal afectó a todos los grupos de forma

generalizada sin diferencias, dificultando la correcta evaluación de los candidatos vacunales.

Por último, el tercer estudio (capítulo VI) examina la inmunogenicidad y eficacia de dos proteínas de la pared celular de *S. suis*: los antígenos MRP2 y C05. Lechones neonatos fueron doblemente vacunados con ambos antígenos en combinación con los adyuvantes CAF®01 o CDA e infectados por vía intranasal con una cepa de serotipo 2 de *S. suis*. Ambas combinaciones produjeron una inmunogenicidad débil, tanto humoral como celular, incapaz de proteger a los animales frente a la infección en comparación con los animales inmunizados con autovacuna.

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López-Serrano S, Mahmmod YS, Christensen D, Ebensen T, Guzman CA, Rodríguez F, Segalés J, Aragón V. *Immune responses in piglets following neonatal vaccination with CAF®01 or CDA adjuvanted conserved fragment F4 against virulent Glaesserella parasuis and the effects observed on its natural colonization.* In preparation.

López-Serrano S, Vreman S, Zwart R, Ruuls L, Fitjen H, Winkelman H, Wells JM, Christensen D, Ebensen T, Guzmán CA, Segalés J, Aragón V, Stockhofe-Zurwieden N. *Immune responses in new-born piglets after immunization with Streptococcus suis surface-conserved antigens combined with CAF®01 or CDA.* In preparation.

Chapter I: General Introduction

Chapter 1. General introduction

Infectious diseases are currently the second leading cause of death worldwide in humans and signify a major threat to wildlife and livestock [1]. In swine, infectious diseases represent a major global problem not only for the pork industry, where it causes a significant welfare and economic burden but also can pose a risk for humans because of the possible emergence of zoonotic diseases. In the context of recent pandemics, climate change and antimicrobial resistance, the concern about the occurrence of new and more virulent infectious diseases is increasing [2]. Albeit vertebrates possess a complex immune system capable of dealing with diverse pathogens, the virulence of some pathogens forces to look for effective strategies to strengthen immunity and tackle them.

In these regards, since the eighteenth century, vaccines have become the most successful and promising tool to control or even eradicate infectious diseases as occurred with smallpox for humans or rinderpest in cattle [3–5]. For the development of new vaccine formulations, pre-clinical vaccination studies are needed to guarantee vaccine safety and efficacy and characterize the triggered immune response and guarantee their safety. Animal model experimentation become an essential procedure in vaccinology, especially the analysis after a controlled infection, indispensable to examine the response along with the mechanisms underlying protection.

The pig is recognized as one of the best animal models not only for the study of porcine diseases but also for biomedical research thanks to their similarity to humans in terms of anatomy and immunity [6]. In addition, young pigs are susceptible to pathogens that can cause systemic diseases such as *Glaesserella parasuis or* zoonotic pathogens like Influenza virus or *Streptococcus suis* [7–9]. These three pathogens represent models of diseases that may help to understand the immunity behind new vaccine formulations at younger ages.

The scarcity of current broad and cross-protecting vaccines against many diseases has prompted the research on subunit vaccines composed of surface-exposed conserved antigens [10,11]. However, peptides and protein fragments often lack intrinsic immunogenicity, and they need to be included in vaccines together with adjuvants. Subunit vaccine combinations can be formulated with one or more antigens and adjuvants, a strategy that will mostly depend on the pathogen and the desired immune response. The characteristics and composition of the antigens become important issues to be considered when selecting a suitable adjuvant combination able an effective immune response.

The immune response against pathogens in swine

The function of the immune system is to protect the organism against infectious diseases. In the pig, as in all vertebrates, the immune system consists of two functional mechanisms that constitute two lines of defence, the innate and the adaptive immune systems [12,13]. The innate immunity is the non-specific response to pathogens, is quicker and can cope with the pathogens to a limited extent. The adaptive immune system on the other hand presents antigen specificity and can generate memory efficiently to prevent further infections. The understanding of host-pathogen interaction is crucial to design vaccine formulations capable of strengthening the immune system and therefore tackle the infections [14].

Innate immune response

The physical barrier constituted by the epithelia, and the molecules secreted by their cells, such as mucus, collectins, defensins and other innate immunity mechanisms comprise the first line of defence against pathogens [15]. Once this barrier is breached, and the cells become damaged or infected, the mechanisms of the innate response are activated. Infected and sentinel cells, such as macrophages and dendritic cells, sense pathogens through the pattern recognition receptors (PRRs), which in turn recognize repetitive conserved motifs present in pathogens, the so-called pathogen-associated molecular patterns (PAMPs) [16]. There are different PRRs depending on its location within the cell (surface, endosomal or cytosolic) and their

ligand (Figure 1.1). The combination of different stimulations triggers the activation of transcription factors, inflammasomes and NF-κB transcription factor which initiates the synthesis and secretion of interferons (IFN) and proinflammatory cytokines such as IL-1, IL-6 and TNF-α [17]. This cytokine and chemokine environment influences the subsequent adaptive immune response. This mechanism is exploited by vaccinologists to increase the immunogenicity and effectiveness of the formulations [18]. Complement pathways can also be triggered by the pathogen, allowing their direct destruction (in the case of some bacteria) or engulfment by phagocytic cells, such as neutrophils and macrophages [19]. On the other side, the NK cells, a subset of innate cytotoxic lymphoid cells, mediate the targeting and lysis of virus-infected cells that express viral molecules on their surface [20].

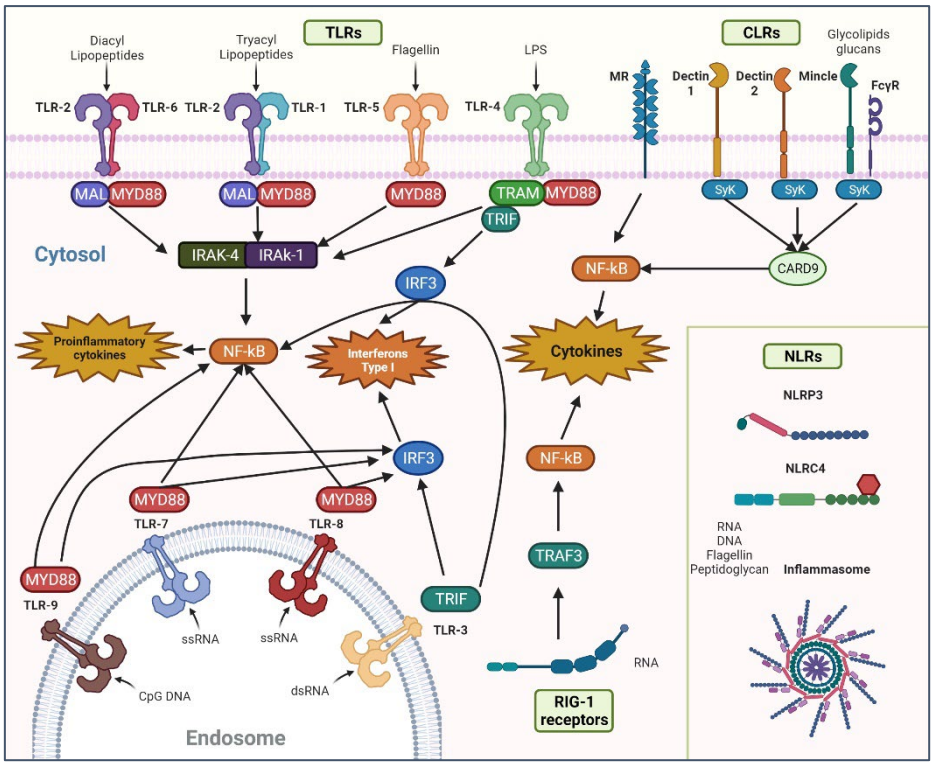

Figure 1.1. Schematic representation depicting the main types of PRRs of the innate immune system, their ligands and the signalling pathways that lead to the secretion of interferons or proinflammatory cytokines. Acronyms for PRRs and surface receptors: C-type Lectin receptors (CLRs), Macrophage inducible Ca^{2+}-dependent lectin receptor (Mincle), Mannose receptor (MR), NOD-like receptors (NLRs), NLR family pyrin domain containing 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), retinoic acid-inducible gene-I-like receptors (RIG-1 receptors), Toll-like Receptors (TLRs). Acronyms for adaptor and signalling molecules: Caspase recruitment domain-containing protein 9 (CARD9), Interleukin-1 receptor-associated kinase (IRAK 1-4), Interferon regulatory factor 3 (IRF3), Spleen tyrosine kinase Syk, MyD88-adapter-like (MAL), Myeloid differentiation primary response 88 (MyD88), Tumour necrosis factor receptor-associated factor (TRAF), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Figure adapted from Netea et al. 2011 [21]*, created with BioRender.com.*

Adaptive immune response

Unlike the innate response, the adaptive response is pathogen-specific and mainly driven by lymphocytes, encompassing humoral and cell-mediated immune responses. Adaptive immunity begins once the antigen presenting cells (APCs) capture pathogens and become activated at the infection site. This activation leads to the further processing of the captured pathogens in fragments and their migration to the draining lymph nodes. Once there, processed antigens can be presented through major histocompatibility complex (MHC)-I or MHC-II to naïve T-cells leading to their activation [22].

The humoral response is mediated by antibodies secreted by B cells, which in the pig express CD21 in their surface, and activated when the organism encounters with the pathogen [23]. The initial antibody response is characterized by the development of a transient IgM response followed by an increasing and robust IgG and IgA responses, promoted by class-switching. Cell-mediated response is mainly supported by cytotoxic T cells, which express CD8 in their surface and become activated through MHC-I antigen presentation. T helper (Th) CD4+ cells regulate the function of both mechanisms (humoral and cell-mediated responses) through the secretion of cytokines that modulate the elicited immune response. The differentiation of naïve T cells depends on different stimulators including the antigen, co-stimulatory molecules and the cytokine milieu secreted by the dendritic and surrounding cells. T helper polarization is orchestrated by different

master transcription factors leading to the corresponding cytokine secretion and immune responses [24,25] (Figure 1.2).

Adaptive immune response is capable to generate memory B and T cells to cope with future infections in a faster manner, and thereby advancing the innate immunity mechanisms. In the pig, memory T-cells express CD4 and CD8 markers on their surface and can be divided into two cell populations according to the expression of CD27. Hence, central memory T cells express CD27+ on their surface, while effector memory T cells not [26].

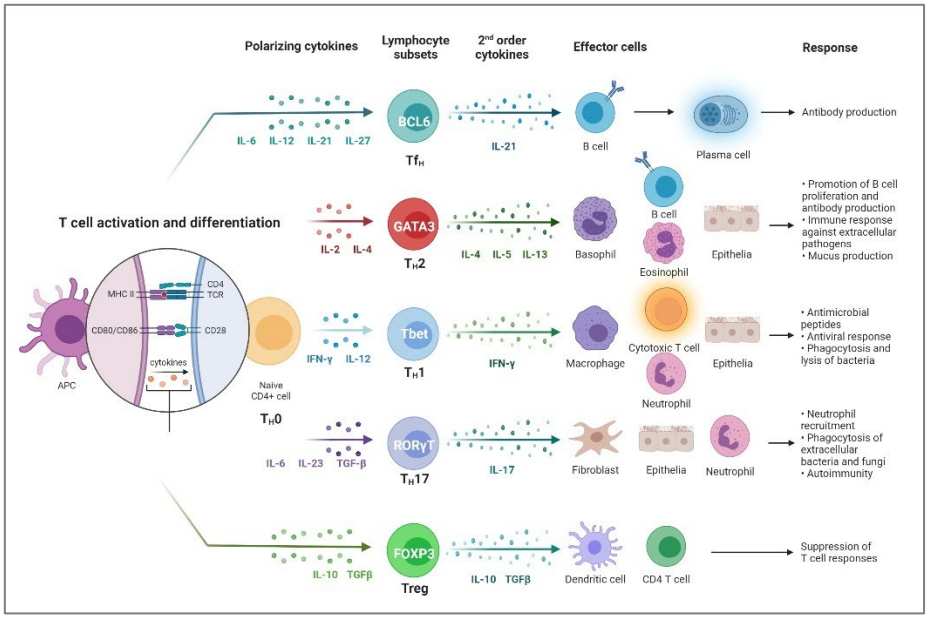

Figure 1.2. T helper (CD4+) cell subset polarization and the subsequent functional responses. Adapted from different sources [24,25]. *Created with BioRender.com.*

Immune response in the neonate pig

The porcine immune system is relatively the most well-characterized after the murine and primate models, with a wide variety of available tools and methodologies [27,28]. Nonetheless, the pig has some particularities that may affect the outcomes of early vaccination. Indeed, passive maternal immunity is acquired through the colostrum and no transplacental transport of antibodies occurs. Colostral IgGs are transferred from the intestinal lumen into the bloodstream of the new-born via enterocytes. [29,30]. Similarly, as it occurs with other mammal species, neonatal immunity is characterized by its low responsiveness to antigens. T helper responses are mainly biased towards Th2 and Treg-type responses, which hinder the T-cell polarization to Th1 or Th17-type responses [31,32].

Porcine diseases studied in this Thesis

Swine production is generally divided into three main periods: gestation/lactation, nursery, and fattening/finishing [33], besides maternity. After 3-4 weeks with their mothers, piglets are weaned, and litters of different sows are commingled together until they reach the grower phase (around 8-11 weeks-of-age). In the nursery phase (post-weaning), piglets are exposed to environmental, social, and behavioural stressors [34]. All these factors, together with a decrease of the maternally derived immunity, impact their health status and predispose to multiple diseases.

One important route of pathogen entry is the respiratory tract. Thus, weaned piglets can be infected by primary bacterial pathogens such as *G. parasuis* or *S. suis*. Albeit the entrance route of these bacteria is respiratory, they may cause Glässer's and streptococcal diseases, two systemic disorders that can severely compromise their health.

Fattening pigs can also be susceptible of swine influenza virus (SwIV) infection as a primary pathogen causing respiratory sickness. Moreover, SwIV can predispose to suffer of a multifactorial syndrome called Porcine Respiratory Disease Complex (PRDC), which is described as clinical respiratory signs associated to pneumonia caused by the interaction of environmental factors, viruses, and bacteria. Indeed, the aetiology of PRDC comprises primary pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), SwIV, porcine circovirus 2 (PCV-2), *Mycoplasma hyopneumoniae* or *Actinobacillus pleuropneumoniae,* as well as other concomitant opportunistic pathogens,

as for instance bacteria from *Bordetella* or *Pasteurella* genera [35]. The economic losses derived from the high morbidity and costs of the control measures, force to look for new and cost-effective strategies to combat these diseases. Among the pathogens potentially involved in PRDC are SwIV and *G. parasuis* [36], tackled in this Thesis. *S. suis* is another bacterium can also be isolated from the lungs of PRDC affected animals, as well as from cases of polyserositis/polyarthritis. Even though it affects severely young piglets as previously mentioned, its association with PRDC is considered questionable since pneumonia does not figure as one of the main clinical features of streptococcal disease [37].

Influenza

Influenza viruses (IVs) are enveloped, negative-sense and single-stranded RNA (-ssRNA) viruses from the *Orthomyxoviridae* family. IVs comprise several genera (named A, B, C and D) that infect mammals and birds, including wild and domestic animals like poultry and pigs, causing the zoonotic respiratory disease called influenza [38].

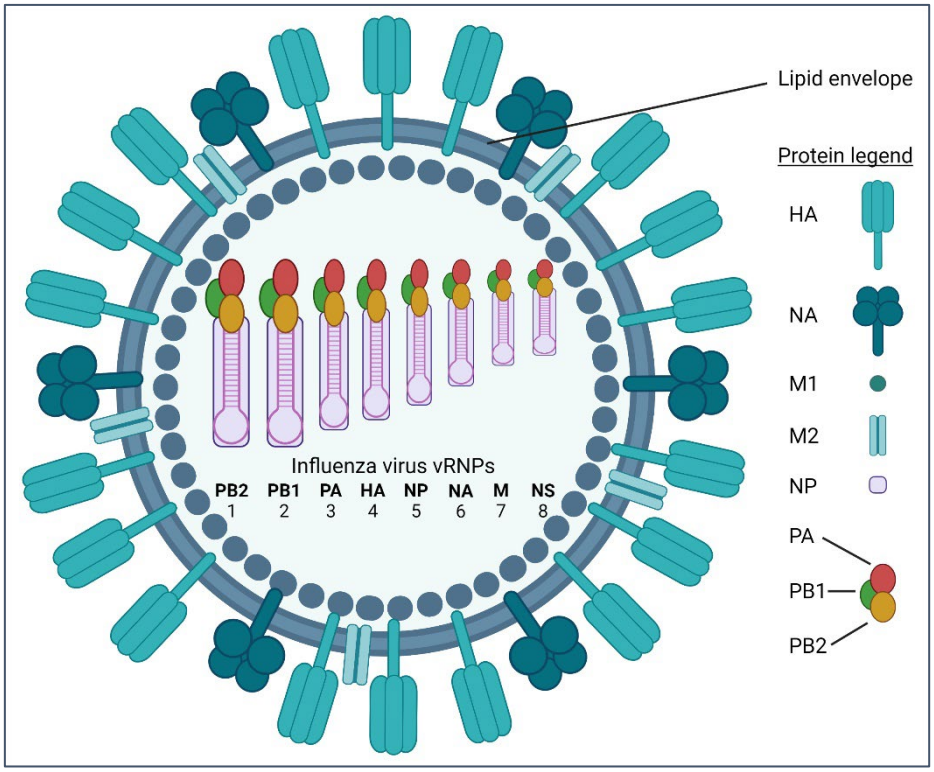

Figure 1.3. Schematic representation of an Influenza virion with the enveloped particle composed of HA, NA, M1, M2 and NP proteins, containing the 8 coding vRNPs and polymerase proteins. Scheme adapted from Krammer et al 2018 [38]*, created with BioRender.com.*

Influenza A viruses (IAVs) are classified by subtypes according to the composition of two proteins located in their envelope: the hemagglutinin (HA) and the neuraminidase (NA). The genome of Influenza viruses consists of 8 coding RNA fragments packed in ribonucleoproteins (vRNPs). Besides HA and NA proteins, there are three fragments coding for polymerase subunits (PA, PB1, PB2), nucleoprotein (NP), matrix proteins (M) and non-structural proteins (NS) [38] (Figure 1.3A). In this genetic composition resides the highly

mutagenic and varying nature of IVs, characterized by the antigenic shift and drift, which help IAVs to adapt their fitness and replicate into different hosts evading the immune system by the reassortment of genome fragments or by point mutations [39].

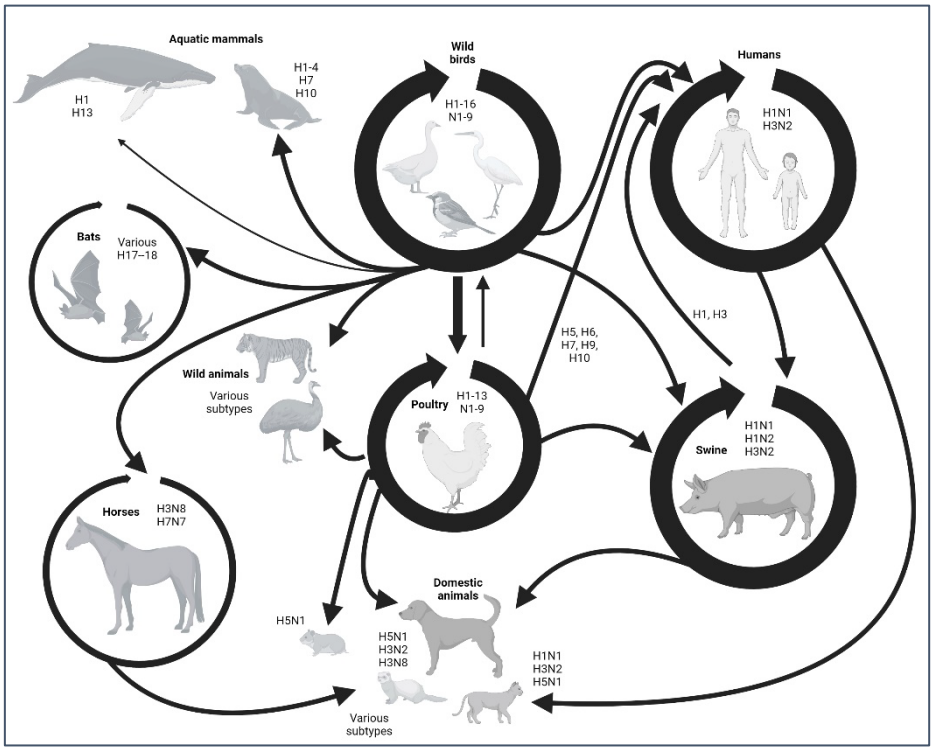

Figure 1.4. Ecology of Influenza A viruses genus depicting the main subtypes affecting the natural reservoirs (wild birds), natural hosts (poultry, swine and humans) as well as other secondary hosts. Adapted from Long et al. 2019 [40].

Nowadays, influenza is one of the main endemic zoonotic diseases of concern for humans, affecting the population seasonally and killing 650,000 people every year (WHO, 2017 report). Wild birds are considered the natural reservoir of IVs, whereas pigs, humans, poultry

and other animals can act as natural hosts [41] (Figure 1.4). Three main subtypes of IAVs H1N1, H1N2 and H3N2 are responsible of the majority of influenza infections in pigs [42]. In swine, the morbidity of influenza is high arriving (up to 100%) but the mortality is considered low (variable but can be even lower than 1%). Nonetheless, outbreaks of Influenza on farms cause significant economic losses for the swine industry because the infection can predispose to concomitant secondary pathogens, either viral or bacterial [43].

In mammals, IVs produce an acute infection of the respiratory tract, while in birds cause a systemic disease. Viral replication in mammals takes place in the epithelial cells lining the airways and alveoli (Figure 1.5). These cells possess in their surface the sialic acid residues and represent the receptors for the IAVs binding to epithelial cells [44]. In this respect, pigs can harbour different subtypes of IAVs and may act as mixing vessels, with α2,6-linked (avian-like) sialic acid residues to dominating in the upper and α2,3-linkages (human-like) in the lower respiratory tracts [45]. The infection of alveolar cells activates macrophages, inducing the secretion of proinflammatory cytokines IL-1β, IL-6 and [46]. Viral replication and TNF-α production will determine the pathophysiology of the disease [47]. The generated cytokine milieu promotes the infiltration of immune cells, neutrophils and NK into the respiratory tissue which will help in the pathogen clearance [48]. In parallel, dendritic cells located below the respiratory epithelia will trigger the adaptative immunity by the further antigen presentation and activation of CTL, B lymphocytes, Th1 and $\gamma\delta$ T-cells, related to cross-protective responses [49].

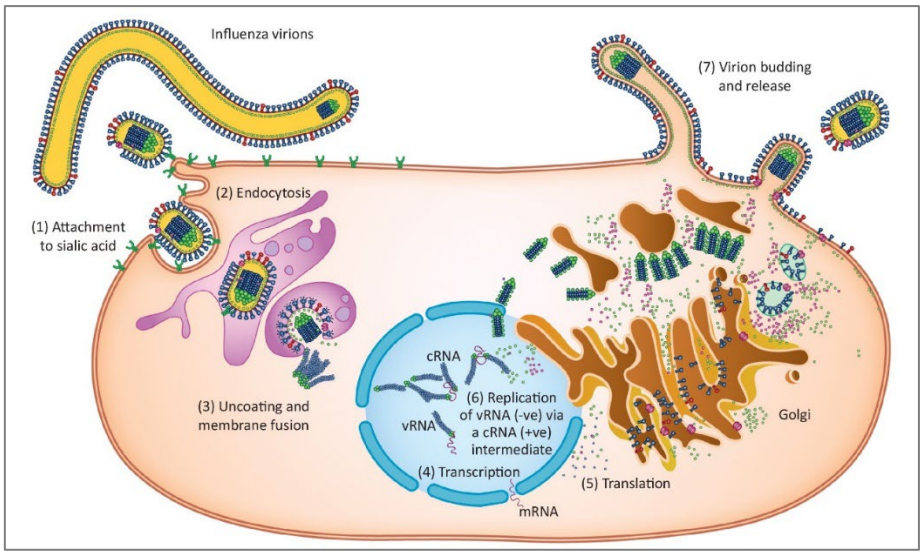

Figure 1.5. Schematic view of influenza virus replication. (1) Spherical or filamentous Influenza virions attach to the host cells by the interaction between hemagglutinin and sialic acid residues located in the plasmatic membrane. (2) Virions are endocytosed by the host cell initiating the infection. (3) Hemagglutinin attachment allows the membrane fusion and the uncoating of the virion, releasing its content into the cytosol. (4) -ssRNA fragments are relocated inside the nucleus where transcription and replication (6) are carried out in parallel. (5) The translation of the membrane proteins is performed in the endoplasmic reticulum and transported by the Golgi network, whereas the soluble proteins are translated in the cytosol. (7) Viral proteins and -ssRNA fragments are packed allowing the virion budding and subsequent release. Figure adapted from Hutchinson 2018 [50].

In swine, classical SwIV infections cause limited clinical signs with a fast onset of 1-3 days. Under natural conditions, SwIV infection starts with a rise of the rectal temperature, including peaks of fever above 40.5ºC, followed by lethargy, nasal discharge, anorexia, dyspnoea, and pneumonia that may generally be resolved after five to ten days [51]. Lesions are generally mild and purulent secretions can be observed in the nasal cavity. At *post-mortem* examination, diseased animals show

mononuclear inflammation of the bronchi and bronchioles as well as hyperplasia of peribronchiolar lymphoid tissue, sometimes with neutrophil infiltration in the lung parenchyma [52]. These lesions are visible macroscopically with the characteristic multifocal cranio-ventral pulmonary consolidations with sharp margins located in the upper lobes and lung portions. Good husbandry practices represent one of the important biosecurity measures to avoid or mitigate the effects of IV infection together with vaccination in pig herds. However, the variability of circulating strains diminishes the effectivity of current vaccines.

Glässer's disease

Glaesserella parasuis is a pleomorphic Gram-negative bacterium from the *Pasteurellaceae* family. It is one of the early colonizers of the upper respiratory tract of piglets, only colonizes suids, and it is ubiquitously distributed worldwide. *G. parasuis* is a highly heterogeneous species classified in 15 serovars, comprising both commensal and virulent strains. Virulent *G. parasuis* strains may cause fibrinous polyserositis in young pigs, which is the hallmark of Glässer's disease, a disorder especially prevalent during the nursery period when the piglets from different litters are commingled together [7]. Glässer's disease has a significant impact on the pig industry, especially during the nursery and early fattening periods. Outbreaks of Glasser's disease can cause mortality between 5-10%, together with poor welfare, and resulting in high economic losses.

G. parasuis colonization of the nasal mucosae occurs in the early stages of life by direct contact with the sow and other piglets [53]. The mechanism of pathogenesis is believed to be multifactorial, and several virulence factors have been proposed, including capsule and diverse surface proteins [54]. Non-virulent strains are normally contained in the upper respiratory tract since they are reported to be sensitive to phagocytosis and complement [55,56]. Non-virulent strains are detected by the innate immune system and the subsequent moderate cytokine release is probably involved in the triggering of an adequate immune response that leads to bacterial clearance. Virulent strains, on the contrary, are resistant to phagocytosis [56]. The particular composition of the outer membrane and the presence of a capsule may help the bacteria being undetectable in the lower respiratory tract, favouring a delayed immune activation that permits bacterial multiplication and the subsequent disruption of the endothelial layer and apoptosis of cells [57,58]. Once they enter the bloodstream, the resistance to the complement system helps the bacteria to survive and replicate [55]. This leads to severe systemic inflammation through the release of proinflammatory cytokines, IL-8 and soluble CD163 [59]. Blood vessel permeability is affected by inflammation, and fibrinogen and other plasma components of blood are released into the body cavities. Is in these compartments where the bacteria can activate the coagulation cascade [60], forming the characteristic fibrin deposits of Glässer's disease in body cavities, synovia and meninges (Figure 1.6).

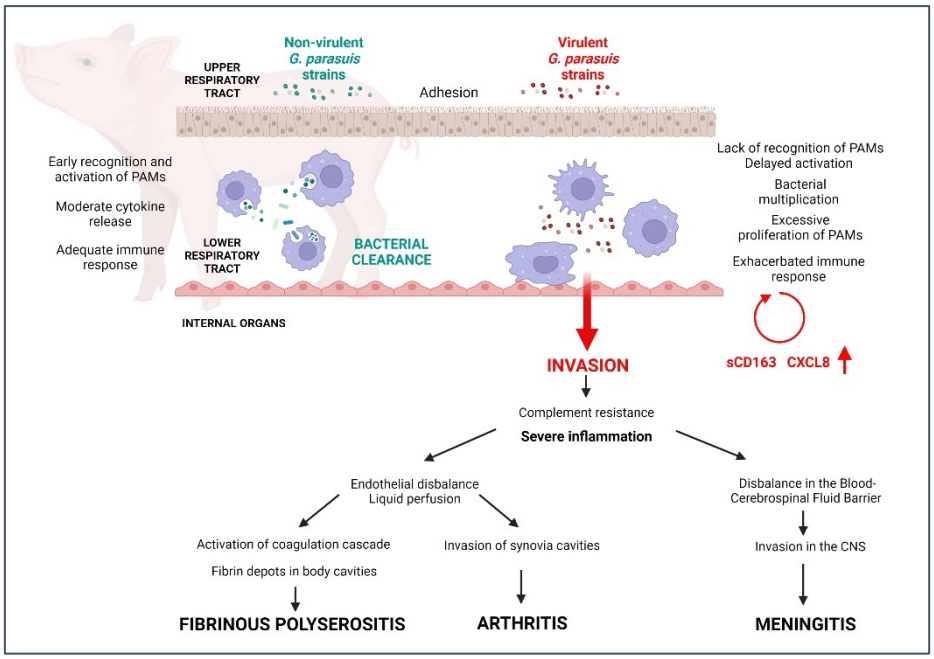

Figure 1.6. Putative mechanisms of Glaesserella parasuis pathogenesis. Adapted from Aragón et al. 2019 [7]*. Created with BioRender.com.*

The onset of the disease has been observed, under experimental conditions, between 24h to 4-5 days after intranasal inoculation with virulent strains in susceptible 4 to 8-week-old piglets. Animals with typical signs of the disease show rectal temperature over 41.5ºC, coughs, abdominal breathing, lameness, and eventual neurological signs [61]. Three forms of *G. parasuis* associated disease have been described according to the generated pathology. Peracute cases of infection present a short course with sudden deaths of less than 48h, without apparent gross lesions but an increase of liquid in the body cavities. Acute cases are characterized by fibrinopurulent exudates in the pleura, peritoneum, pericardium, and synovia observed *post-mortem*, and correspond to the typical pathology of Glässer's disease [62]. In some

cases, animals can overcome the infection and acquire a chronic state of the disease with reduced growth rates and fibrosis in the body cavities. As occurring with other bacterial diseases, Glässer's disease can be prevented using antimicrobials. However, these treatments are nowadays controversial due to the arising concern of antibiotic resistance in livestock and are starting to be restricted. Thus, the search for new strategies to prevent Glässer's disease is focused on vaccines.

Streptococcal disease

Streptococcus suis is an encapsulated, facultative anaerobic, Gram-positive bacterium from the *Streptococcaceae* family. It is an early colonizer of the upper respiratory tract of swine, inhabiting predominantly the tonsils and nasal cavities of pigs at any age, although also found in digestive and genitourinary mucosae. *S. suis* comprises 29 serovars that can be differentiated into three groups, highly pathogenic, weakly pathogenic and avirulent [63]. Pathogenic strains may cause systemic disease, mainly in weaned piglets, with the clinical features of septicaemia and meningitis [64]. Piglets become naturally colonized through the birth canal and transmission between individuals can be produced by direct contact [65]. The majority of strains isolated from diseased domestic pigs belong to serovars 1 to 9, being the serovar 2 the most prevalent worldwide in the western hemisphere and the most concerning because of its virulence and zoonotic potential [9]. *S. suis* is also considered an emerging zoonotic pathogen, as it may infect humans through direct contact, open wounds, or consumption of

infected raw pig products. The disease in humans is characterized by headache, fever, and nervous signs, as the main clinical manifestation of meningitis (Figure 1.7). Disease outbreaks of human streptococcal disease have been reported in Asia with high rates of fatalities [66]. *S. suis* is a ubiquitous bacterium present worldwide and it is becoming one of the most important pathogens affecting pigs, responsible for poor welfare of the animals, excess of antimicrobial use and economic losses in the swine industry. The streptococcal disease affects predominantly pigs between 5-10 weeks of age with high mortality rates without treatment (>20%).

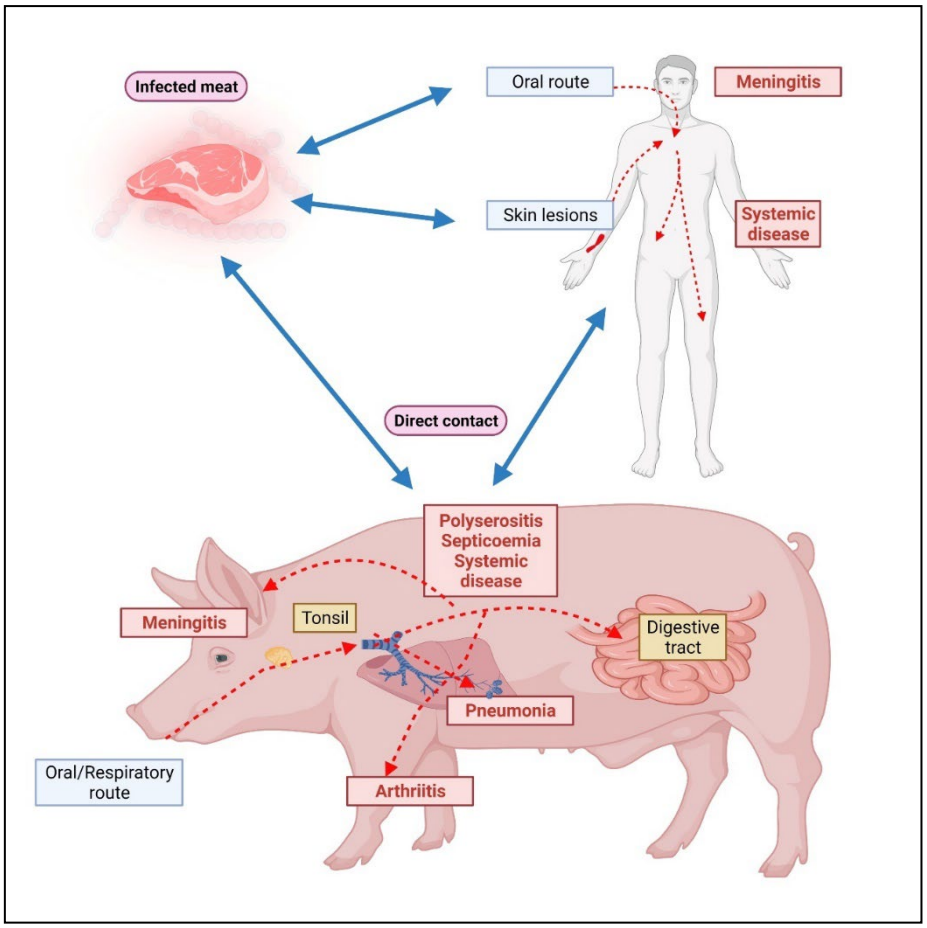

Figure 1.7. Epidemiologic and pathologic features caused by Streptococcus suis in swine and humans. Adapted from Gottschalk and Segura 2019 [64].

The pathogenesis of *S. suis* remains mostly unknown. A myriad of virulence factors have been described so far, and in general it is agreed that is a multifactorial process (Figure 1.8) [67]. Based on experimental studies, it was established that the main entrance route is the respiratory system. Virulence factors such as the capsular polysaccharide (CPS) and the haemolysin Suylisin (SLY), together with other virulence markers such as the muramidase-released protein (MRP) and the extracellular factor (EF), are the most studied ones [68,69]. The process by which *S.*

suis can damage the mucosa is not well understood. It seems that CPS together with multiple adhesins mediates the attachment and subsequent invasion of the host cells [70]. The interaction of the cell-wall proteins with the host cells triggers the production of proinflammatory cytokines and possible apoptosis in the epithelial tissue allowing the subsequent invasion [71]. The innate immune response against *S. suis* early in life seems to be modulated by the strain virulence [72]. Once the pathogen has reached the bloodstream, mediated by the CPS, SLY and other proteins, *S. suis* can evade the immune system hampering the alternative complement fixation and phagocytosis mediated by neutrophils and macrophages [73]. The adhesion to the endothelial cells of the choroid plexus produces a disbalance in the cerebrospinal barrier [74]. This characteristic helps the bacteria entering the central nervous system, producing meningitis. Furthermore, the septicaemia and severe inflammation produce a generalized disbalance of the endothelial tissues, leading to a diffusion of plasmatic components and triggering the coagulation cascade with the typical outcomes of fibrinous polyserositis. Moreover, *S. suis* possess different mechanisms to evade host-defence (Figure 1.8) [75].

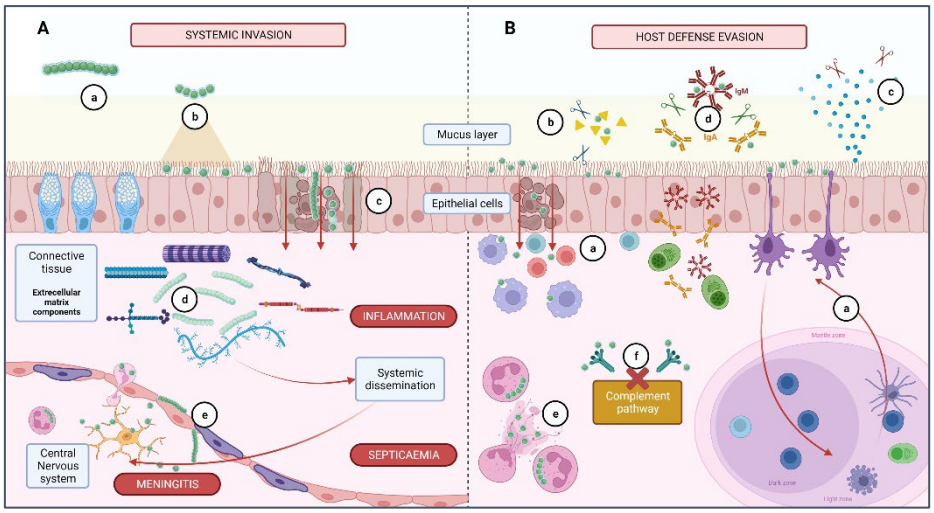

Figure 1.8. Pathogenicity of S. suis. ***(A)*** *Mechanisms of systemic invasion. (a) S. suis can adhere and cleave the mucin of the mucus layer. (b) Several surface proteins with adhesion properties help the bacteria to adhere to the host cells, adhesins, toxins and other cell wall enzymes are involved in this process. (c) The process of invasion of the epithelia is largely unknown, proteases and suilysin may play a role in inducing damage to cell integrity helping their traverse across the cells. (d) S. suis can adhere to multiple proteins located in the extracellular matrix of connective tissues promoting the invasion of the submucosa and therefore the system. (e) Once in the bloodstream, S. suis can adhere to the endothelial cells of the choroid plexus and blood brain barrier destabilizing the tight junctions and allowing its entrance; additionally, S. suis can survive in acidic environments like the phagosomes taking profit of phagocytic cells as trojan horses.* ***(B)*** *Mechanisms involved in the immune recognition of S. suis and evasion of host defence. (a) S. suis is sensed by multiple immune cells like macrophages, T cells, B cells and dendritic cells, allowing to a trafficking between lymph nodes to epithelia. (b) Cleavage of defensins and other innate immune proteins. (c) Cleavage of chemokines like IL-8 that hampers the recruitment of further immune cells. (d) Secretion of IgA and IgM proteases. (e) Protection against the neutrophil activity. (f) Mechanism to resist, cleave or inhibit the complement pathways. Adapted from Segura et al. 2016* [67] *and Doran et al.* [76]. *Created with BioRender.com.*

The acute form of the disease is characterized by rise of the rectal temperature above 40ºC. Shortly after, animals may develop nervous signs due to meningitis, whereas dyspnoea and lameness caused by polyserositis, and arthritis are often observed. Pneumonia might appear but is far less common, and its pathogenesis is not clear at all. Infected animals can also develop a peracute disease with sudden deaths caused by septic shock without premonitory signs. In some cases, animals may overcome the acute infection and develop a chronic form of the disease presenting reduced average weight [64]. Current prevention and control of the disease rely on the good husbandry of the animals, antimicrobials, and autogenous vaccines.

Vaccines

Definition of vaccines and types

Vaccines are nowadays considered the most powerful tool for the prevention and control of infectious diseases, contributing to the health, and general well-being of humans and animals since their development. Animal and human vaccines share a common history, in fact the term vaccine was coined at the end of the 18th century by the British physician Edward Jenner referring to *Variola vaccinia*, the cowpox infection that comes from the latin name of the cow, *vacca.* Vaccines are based and defined conceptually on the stimulation of the body's immune response by the inoculation of an exogenous substance, generally a weakened or a portion of a pathogen, that can confer immunity to the host against the infection by the same pathogen [77]. Vaccines are classified into different types according to the nature and complexity of the antigen used for immunization (Table 1.1).

Table 1.1. Main types of current licensed vaccines.

Type	Immunogen	Immunogenicity	Advantages	Disadvantages
Inactivated	Heat or chemically killed pathogen	Weak	• Safety • Economic	• Lack of strong immunogenicity • Need for periodic immunizations • Sometimes formulated with adjuvants
Live-attenuated	Weakened pathogen	Strong	• Long- lasting immune response • Few doses	• Risk of pathogenic reversion • Special handling (refrigeration)
Toxoid	Inactivated toxin	Strong	• Economic	• Booster doses
Subunit / conjugate	Protein fragment of the pathogen	Weak	• Safety	• Lack of intrinsic immunogenicity • Need of adjuvants • Booster doses
DNA/RNA based	Nucleic acid sequence	Strong	• Safety • Lack of risk of pathogenicity	• Low stability. • Special handling • Difficult management
Viral-vectored	Use of a virus as a vector	Strong	• Management immunogenicity	• Adverse effects

Influenza virus vaccines

Vaccination represents the main tool for prevention of IVs infections, contributing to reduce havoc and economic losses that the illness can produce in both human and animals. The efficacy of flu vaccines is measured by their capacity to elicit neutralizing antibodies [78].

Nonetheless, the variability of strains makes the production of efficient vaccines a big challenge to manufacturers.

Every year, according to the World Health Organization (WHO) directives, composition of licensed seasonal influenza vaccines for human use are changed based on the main subtypes of the seasonally circulating strains. Thus, the composition of available human influenza vaccines can be trivalent with H1N1 and H3N2 subtypes from IAV and one influenza B virus (IBV) strain, or quadrivalent when contains both lineages (Victoria and Yamagata) of IBV, since IBV strains also affects human. Most of the seasonal influenza vaccines consist of unadjuvanted formulations containing purified split virions [79]. The efficacy of seasonal influenza vaccines varies every year depending mainly on the coincidence of its composition to the circulating strains. For this reason, weakly paired preparations may deliver mild or not protection against circulating strains.

Influenza vaccines for swine predominantly consist of inactivated virus formulations produced in embryonated eggs or cell cultures. Unlike human vaccines, composition of commercialized swine preparations contain adjuvants and are not standardized every season due to a lower variation of SwIV in comparison to its human counterparts [80]. Current SwIV vaccines are administered by intramuscular route and its composition can contain H1N1 and H3N2 subtypes (bivalent) or H1N1, H1N2, H3N2 subtypes (trivalent). In fact, the strain composition of each vaccine may differ depending on the manufacturer's criteria according to the circulating strains either in Europe or North America [81,82].

Unfortunately, the efficacy of SwIV vaccines is moderate at its best, since they partially protect against heterologous strains.

Glässer's disease vaccines

Nowadays, commercial vaccines against Glässer's disease consist of formalin-killed *G. parasuis*. These formulations, which are adjuvanted, may include one or more serovars and are efficient in producing opsonizing antibodies, preventing in some cases the onset of the disease [83]. Unfortunately, they do not elicit cross-protection against heterologous strains and require multiple immunizations. Similarly, another approach to vaccination of animals used in farms is the application of autogenous vaccines, although their use is controversial due to safety and efficacy reasons. Different approaches consisting of surface proteins are being tested [84,85].

S. suis vaccines

Unlike *G. parasuis*, there are no commercial vaccines against *S. suis*. Available preparations against *S. suis* are limited to autogenous vaccines that cannot provide cross-protection against diverse virulent serotypes. Several candidates, including inactivated or subunit immunogens, have been tested under experimental conditions, but the complexity of *S. suis* pathogenesis and the diversity in virulence factors makes the search for effective vaccines a big challenge [86].

In all the above cases, current vaccines either commercial or autogenous confer limited protection against different strains or subtypes. For this reason, ongoing research has focused on subunit immunogens based in conserved sequences of surface-exposed proteins. This approach should help developing optimal vaccines that may confer cross-protecting immunity against different serovars.

Immunogens used in this Thesis

Conserved hemagglutinin peptide NG34

Influenza hemagglutinin (HA) is a glycoprotein located in the envelope of IAV, being the most abundant and surface-exposed protein of the virion [87]. Eighteen different types of hemagglutinin have been described so far, which help classifying the virus, together with the different types of neuraminidases. HA is a highly variable protein, and punctual changes in its amino acid sequence (antigenic drift) may allow the virus evading the immune system. As its name indicates, HA can agglutinate red blood cells which in turn permits the identification and quantification of the virus as well as to determine neutralizing activity of antibodies through the hemagglutination inhibition assay (HIA) [88].

Integral hemagglutinin consists of a cylinder-shaped homotrimeric protein (HA) structured in two domains: the globular head and the stem. HA in turn is formed by two subunit proteins linked by a disulfide bond: N- and C- terminals of subunit 1 (HA1) together with complete HA2 form the stem of the molecule [89]. The globular head domain contains variable antigenic sites of the protein including the receptor-binding site (RBS) that recognizes sialic acid residues on the host cells and triggers the endocytosis. The stem domain in turn, is well-conserved and has fewer immunogenic sites, mediating the virus-host membrane fusion in the endosome which finally leads to the release of the viral contents into the cytosol [90]. HA show specificity for α2,3-linked (avian) or α2,6-linked (human) sialic residues depending on its subtype.

Veljkovic and colleagues (2009) defined a relatively high conserved region of HA1 through informational spectrum modelling. This region was named VIN1 and is located within the site E of the HA1 N-terminus [91]. Based on the bioinformatic information available, this conserved domain is close to the RBS region and has an important role in the interaction virus–receptor, representing a potential target for the generation of vaccine candidates (Figure 1.9). A mixture of five predicted peptides derived from this VIN1 region, including one peptide derived from the pandemic H1N1 IV, called NF-34, were adjuvanted with Freund's adjuvant and used as immunogens in a vaccination study with pigs. Vaccinated animals elicited a strong humoral response as well as an increase of IFN-γ secreting cells after NF-34 recalling in vitro [92]. After the challenge, partial protection was observed in immunized pigs that did not correlate with the detected neutralising antibodies.

Table 1.2. Indicative table of the origin and sequence from influenza virus conserved immunogenic peptides originated from the conserved VIN1 region of hemagglutinin.

Peptides	Virus origin	Sequence
NF34	A/South Carolina/1/18	NS**E**NGTCYPGDFIDYEE LREQLSSVSSFE**K**FEIF
NG34	A/Catalonia/063/2009	NS**D**NGTCYPGDFIDYE ELREQLSSVSSFE**R**FEIF

In bold red are depicted the two amino acid modifications performed to enhance the immunogenicity. Table modified from Sisteré-Oró et al. 2019 [93].

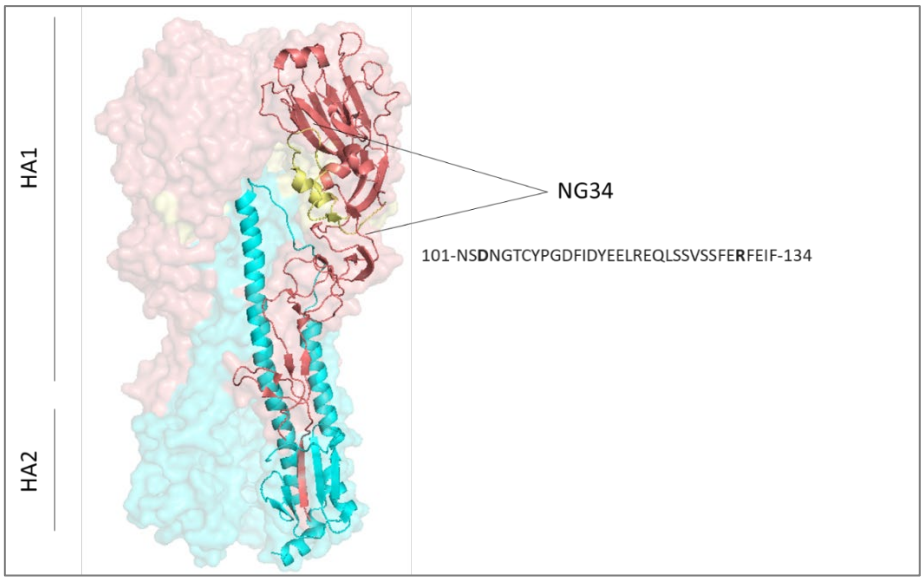

Figure 1.9. Position of the NG34 peptide (in yellow) within the hemagglutinin molecular structure; the HA1 subunit is depicted in red, whereas the HA2 subunit in turquoise blue (PDB no. 7KNA). Graphic performed with PyMOL Molecular Graphics System v4.6, courtesy of Álvaro López-Valiñas.

To improve the level of neutralising antibodies, a new peptide named NG34 was adapted from the homolog sequence of NF34 in the pdmH1N1 A/Human/Catalonia/063/2009 strain (Table 1.2, Figure 1.9). The NG34 peptide conferred neutralising and cross-reactive humoral response correlating with protection in various preliminary experiments using mice and chicken [94]. The NG34 peptide was later formulated in a DNA-based vaccine together with CTLA4 as an adjuvant in a pig experiment, eliciting an anamnestic humoral response with a reduced viral shedding after a heterologous challenge with SwIV H3N2 [95]. Similarly, in another vaccine experiment with chicken, NG34 expressed along with flagellin in a baculovirus system provided significant protection and reduction of flu-like clinical signs after a

heterologous challenge with highly pathogenic avian influenza virus (HPAIV) of H7N1 subtype [96]. Finally, a comparative study of different adjuvants (Aluminium hydroxide, MF-59 analogue, and CAF®01) combined with NG34 was performed in mice to define an immune efficient combination to explore in larger animal models. Among all the combinations, only NG34 formulated with CAF®01 induced a complete immune response with high titres of IgG1, IgG2c and high percentages of activated T-cells biased to Th1/17-type responses [97].

Conserved VtaA fragment F4

Trimeric autotransporters are a wide family of outer membrane proteins involved in adhesion and virulence described in most pathogenic Gram-negative bacteria [98]. Trimeric-autotransporters from *G. parasuis* were firstly described in 2009 by Pina et al. [99] and named *virulence-associated trimeric autotransporters (VtaA)*. These transmembrane surface-exposed proteins comprise of *G. parasuis* proteins of different lengths, but sharing a similar structure defined in three main parts: an N-terminal leader peptide, a passenger domain with characteristic motifs of adhesins, hemagglutinins and repeated collagen motifs, and a C-terminal domain of anchorage to the outer membrane. Thirteen VtaAs were identified in the virulent strain of serovar 5 Nagasaki. *In vitro* experiments confirmed the function of VtaA 2 in adhesion to the host extracellular proteins [100] and VtaA 8 and 9 in phagocytosis resistance [101]. VtaA antigenicity was also confirmed by the presence of anti-VtaA antibodies in convalescent piglets, indicating its immunogenic

potential as a candidate for vaccine development against Glässer's disease [102]. Moreover, whole-genome studies of diverse strains of *G. parasuis* detected differences in the signal sequence of the *vtaA* genes between virulent and non-virulent strains [103]. These differences helped developing a diagnostic PCR for the prediction of *G. parasuis* virulence [104].

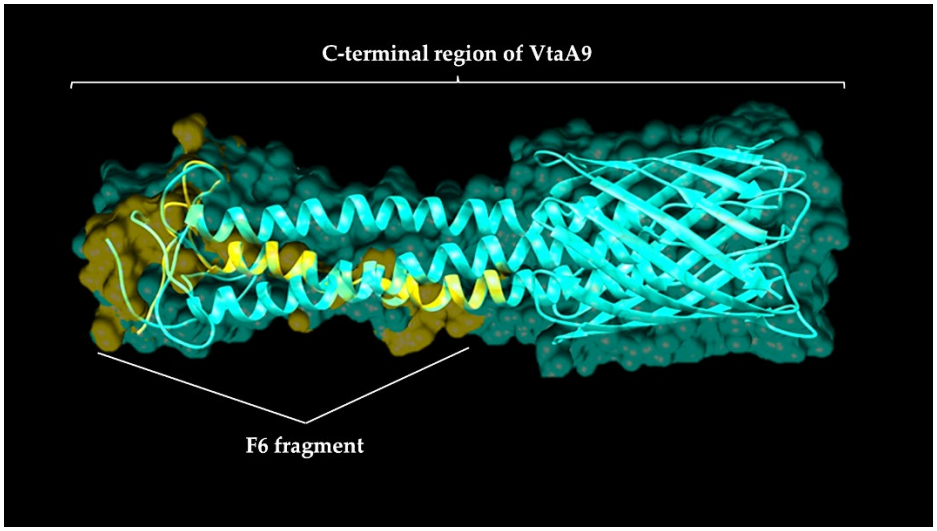

Figure 1.10. Carboxy-terminal region of virulence trimeric autotransporter 9 (vtaA9) from virulent G. parasuis. In yellow is depicted the F6 fragment, which represents the third part of F4 protein fragment and includes determined antigenic epitopes. Picture courtesy of Florencia Correa-Fiz.

Six representative recombinant VtaA proteins that induced antibodies detected in convalescent sera, were tested together in a vaccination experiment in pigs. Immunization with recombinant VtaAs combined with Freund's adjuvants provided an enhanced humoral and mucosal responses in vaccinated piglets and partial protection against a lethal challenge with the homologous strain [105]. Moreover, individual VtaA

vaccination experiments in mice and pigs revealed cross-reaction in animals immunized with VtaA9, including VtaA from 1-8 [106]. Following an in-depth analysis of the VtaA structure and especially the discovery of an opsonic cross-reactive monoclonal antibody produced with VtaAs8 and VtaA9 suggested the presence of a common epitope in the VtaAs of virulent strains [101]. In support, *in silico* prediction studies for the detection of MHC-II epitopes identified five conserved immunogenic sequences located in the carboxy-terminal section of the passenger domain common to the VtaAs from virulent *G. parasuis* [107]. A protein fragment of 134 amino acids, named F4 (Figure 1.10) and conserved in VtaAs from virulent strains, showed reaction with the opsonic cross-reactive monoclonal antibody and lack of collagen domains. Such F4 fragment tested adjuvanted along with carbomer in a sow vaccination study to characterize the effects of this vaccination in their offspring [108]. Besides the expected increase of anti-F4 maternal antibodies, piglets from vaccinated sows had high amounts of TGF-β in blood early in life and high surfactant protein D (SP-D) in broncho-alveolar lavage fluid (BALF) after a challenge with virulent strains of serovars 5 and 13. These results made F4 protein a promising vaccine candidate against Glässer's disease.

Streptococcus suis antigens

MRP2

MRP2 is a fragment of MRP that contains all its variable and surface-exposed regions including a sequence conserved in pathogenic strains. MRPs are a family of wall-anchored proteins of approximately 136 kDa located on the outer surface of the cell wall of *S.* suis (Figure 1.11A). MRP presence is associated with virulence and considered one of the traditional virulence factors of *S. suis*, although its presence is not completely necessary for causing disease [109]. MRP functions are unclear, but it is hypothesized that MRP proteins are involved in the adhesion to fibronectin and host cells, as well as invasion and immune evasion [110].

MRP proteins are supposed to be expressed *in vivo* and secreted into the medium during the infection [111], suggesting their immunogenic interest. Several immunization studies have been performed in mice [110] and swine. Previous immunogenicity studies performed in pigs, immunization with MRP together with EF combined with water-in-oil adjuvant Specol® provided protection against homologous and heterologous lethal challenges with *S. suis* [112]. Animal vaccination with water-in-oil emulsion elicited a strong humoral response that correlated with protection and less severe lesions, unlike antigens adjuvanted with Alum which displayed a diminished immune response. Nonetheless, animals vaccinated with one antigen emulsified with Specol® either MRP or EF did not elicit a protective immune

response indicating the requisite to combine different target antigens for effective immunization.

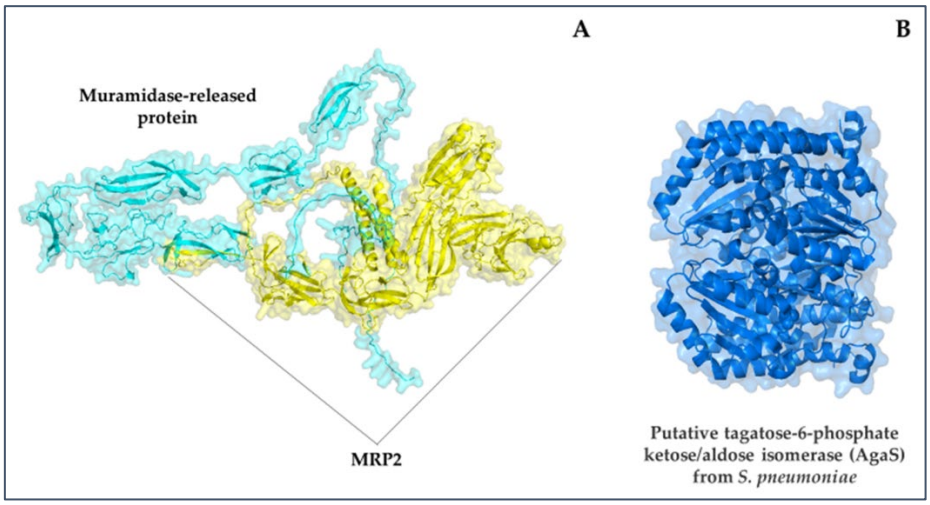

Figure 1.11. Predicted molecular structure of S. suis antigens. (A) Situation of MRP2 antigen (yellow) within a predicted by AlphaFold MRP protein from S. suis (PDB no. AF-P32653-F1). (B) Crystal structure of putative tagatose-6-phosphate ketose/aldose isomerase from S. pneumoniae (PDB no. 3I0Z), which shares an identity percentage of 56.19 to S. suis molecule. Pictures performed with PyMOL Molecular Graphics System v4.6, courtesy of Álvaro López-Valiñas.

C05 antigen

C05 antigen (or SSU0185) is a protein of 432 amino acids (47.2 KDa) from the putative exposed cell wall enzyme AgaS (tagatose-6-phosphate aldose/ketose isomerase) of *S. suis* (Figure 1.11B), presumably involved in the degradation of amino sugars and identified through whole-genome sequencing [113]. C05 was characterized together with another four genes by a high-throughput technique of transposon-directed insertion (TraDIS), directed to identify and select genes encoding proteins critical for colonization. Selected candidates were later used as

immunogens in a vaccine protection study with caesarean-derived colostrum-deprived piglets [114]. The mixture of the selected candidates adjuvanted with an oil-in-water emulsion containing dimethyldioctadecylammonium bromide achieved a protection of 90% against a lethal challenge with virulent serovar 2 of *S. suis*. Besides protection, vaccinated piglets elicited a remarkable humoral response against the proteins in comparison with control groups. Moreover, PBMCs from the vaccinated piglets had an increase of IFN-γ secreting cells and a Th1/Th2 cytokine profiling after boosters. Similar results of survival and humoral response were obtained in mice vaccinated with biopolymer particles containing the same mixture of conserved peptides [115].

Adjuvants

Definition and origin of adjuvants

The term adjuvant comes from the Latin *adjuvare*, which means to help or aid. The term was coined in the 1920s by Gaston Ramon and Alexander Glenny during experimentation with diphtheria and tetanus toxoids in horses. They observed independently that the addition of some exogenous substances in combination with toxin formulations generated higher yields of anti-toxin antibodies [116].

An adjuvant is defined as a substance added to a vaccine that helps triggering an effective immune response, enhancing, and improving its magnitude, breadth, and durability. Adjuvants are used when the antigens used for vaccination are poorly immunogenic, i.e., they lack the intrinsic properties capable to trigger an adequate innate and subsequent adaptive immune responses [117]. Despite its importance for vaccine progress, adjuvants had historically a slow development due to safety reasons [118].

The role of adjuvants in vaccine formulations

Adjuvants are considered essential components of vaccines since they should promote effective immune responses and therefore would help in the prevention of the mortality and morbidity caused by infectious diseases. They add reactiveness to vaccine formulations, restoring or improving the immunogenicity of the antigens, with the minimum impact possible on tolerability. Adjuvants can currently be included in

both inactivated or subunit vaccines for human and animal use [119,120].

The selection of an adequate adjuvant must be based in the physicochemical nature of the antigen, the type of the desired immune response, the age of the targeted population and route of administration [121]. Two main reasons are observed to incorporate adjuvants in a vaccine formulation. The first reason aims to increase at maximum the magnitude of the immune response of weak antigens in terms of antibody amounts and seroconversion, but at the same time to facilitate the use of smaller quantities of antigen and reducing the number of doses (dose-sparing) [122]. The second reason to include an adjuvant in a vaccine formulation is the stimulation of the innate immunity to guide it towards a specific adaptive immune response against a desired pathogen [123]. Therefore, the use of adjuvants implies expected benefits such as a rapid immune response against pathogens, its use in not fully immunocompetent subjects (such as elder and neonate individuals), and reduced number of immunizations. In addition, other important aspects to evaluate are the source of origin and its production costs, especially when formulating livestock vaccines where generally lower relation of cost-benefits are expected [124].

Mechanisms of action of adjuvants

Vaccine adjuvants perform their effects through three main mechanisms of action [125,126] (Figure 1.12):

- Antigen depot formation

  Adjuvants such as particulate carriers or emulsions that entrap, aggregate, or adsorb the antigens, form a "depot" in the injected tissue. This depot promotes the retention of the immunogen within the tissue and permits its slow release preventing the degradation or loss through the bloodstream and promoting the antigen intake by local dendritic cells.
- Recruitment of immune cells

  This effect consists of the induction of local proinflammatory response that favours the recruitment and activation of immune cells. However, this inflammatory response can lead to adverse effects such as swelling, redness, and local pain.
- Immune modulation

  The immune modulation can be achieved at different levels. The adjuvants consisting of immunostimulatory molecules, which possess an intrinsic immune activity, are recognized by the PRRs located either in the plasma membrane or inside the cell. The binding of different PRRs trigger the innate immune pathways that leads to the activation of inflammasomes and transcription factors promoting the synthesis of proinflammatory cytokines. Eventually, the generated cytokine milieu influences the subsequent adaptive response. In addition, some adjuvants can enhance the antigen

presentation. Altogether, immunomodulatory molecules help to harness the immune system in favour to derive into a desired immune response shaping the immune profiling triggered by the immunogen.

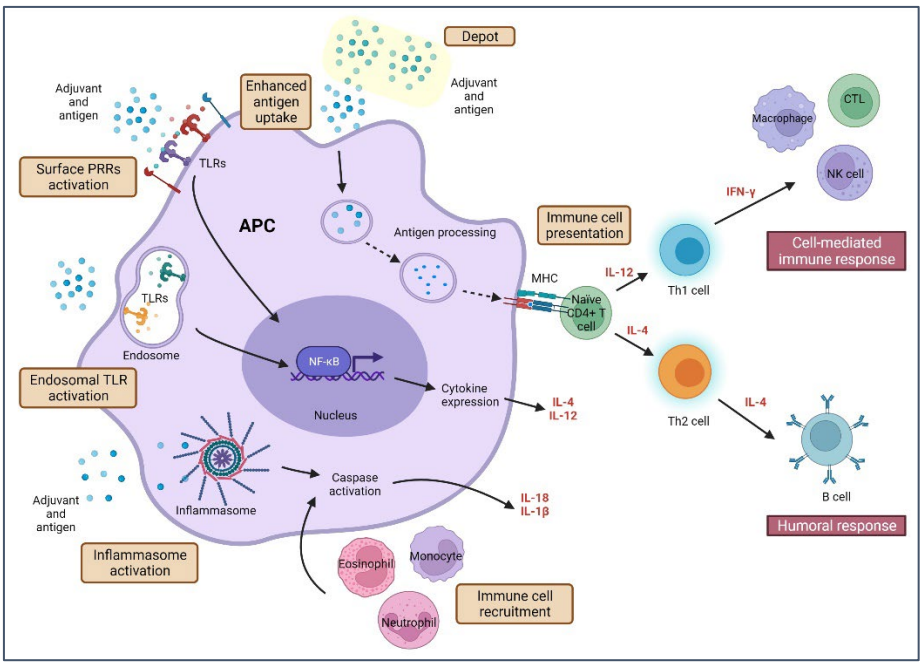

Figure 1.12. Schematic representation of the different mechanisms of action exerted by adjuvants. Adapted from Reed et al. 2013 [127].

Types of adjuvants

Due to the diversity in composition, mechanism of action, triggered immune responses and particularly the diverse combinations used in vaccine formulations, it is difficult to establish a simple classification for adjuvants. In broad terms, vaccine adjuvants can be classified into three main groups: the classical adjuvant molecules, immunostimulatory molecules and adjuvant complexes [128].

Classical adjuvants are also called delivery systems or vehicles because they carry the antigen within the formulation. Once applied, the formulation, which may form a depot or not, can induce a local inflammation in the injection site that allows the recruitment of macrophages, dendritic cells, and B cells facilitating the antigen uptake. Likewise, classical adjuvants conform a heterogeneous group comprising emulsions, aluminium salts, liposomes, polymers, nanoparticles, and other carrier molecules.

Emulsion adjuvants are composed of a mineral oil fraction and a surfactant molecule that generates microdroplets when mixed with the immunogen. Three types of oil emulsions are currently used depending on the physicochemical nature of the antigen. Emulsions generally enhance a robust antibody response and enable the generation of cell-mediated immune responses. On the other hand, particulate carriers transport the antigen in multiple forms as encapsulation, adsorption, or retention. Latest novel formulations adapt this characteristic with the addition of immunostimulatory molecules for enhancing the immunogenicity of the combination. These are the adjuvant complexes and can be considered as a third type of adjuvants (Table 1.3).

Table 1.3. Types of classic adjuvants used in human and animal vaccines with examples.

Adjuvant Type	Composition	Example	Target /Immune response
Emulsions	**Water-in-oil (W/O)**	**Freund's adjuvants** **Specol® (mineral oil mixture)** Montanide™ (W/O)	Th2, humoral response
	Oil-in-water (O/W)	MF59 (Squalene + Mineral oils)	Th2, humoral response
	Saponins	Quil A®, ISCOMs	Th1/2 and humoral responses
Particulate Carriers	Aluminium salts	Alum	Th2, humoral response
	Polymers	Carbomers	Th1 response
Adjuvant complexes	**Liposomes**	**CAF adjuvants** AS adjuvants	The triggered immune response will depend mainly in the immunostimulatory molecule included in the formulation

Adjuvants used in this PhD thesis are pointed in bold. Acronyms: AS (Adjuvant system), CAF (Cationic formulated adjuvants), ISCOMs (immunostimulatory complexes).

Unlike classical adjuvants, immunostimulatory molecules possess an intrinsic immune activity [129]. These compounds are constituted mainly by ligands of the PRRs from innate immune cells that recognize the PAMPs, conserved structures and motifs present in pathogens. The union of PRRs with their ligand, present in the surface of immune cells like APCs and macrophages, triggers signalling pathways that leads to a particular immune response. In turn, immunostimulatory molecules can be classified according to the receptor by which are recognized (TLRs, CLRs or intracellular receptors) [130]. Furthermore, this group of adjuvants can also include cytokines and other compounds (as vitamins) with immunostimulatory properties used in research and some animal vaccine formulations. Examples of immunostimulatory molecules are shown in Table 1.4.

Table 1.4. Examples of immunostimulatory molecules used as adjuvants or within an adjuvant combination.

Type	Receptor	Ligand/Adjuvant	Immune response
Toll-like receptor (TLRs) ligands	TLR1/2	Lipopeptides	Th1/Th2 and CTL responses
	TLR3	Poly-I:C	Th1/Th2 responses
	TLR-4	LPS, MPL (a component of AS04)	Th1 response
	TLR-5	Flagellin	Th1/Th2 responses
	TLR-9	CpG	
C-type lectin receptors	**Mincle**	**Trehalose dibehenate (TDB) (a component of CAF®01)**	**Th1/Th17 and humoral responses**
Intracellular receptor agonists	**STING**	**CDA**	**Th1/Th2/Th17 and humoral responses**
Nod-like receptors (NLRs) ligands	NLRP3	Alum	Th2 responses
Vitamins	**Unknown**	**α-tocopherol**	**Humoral response**
Chemicals	**CD1d (invariant NK T cells)**	**α-Galactosyl ceramide**	**Th1 and humoral responses**

In bold are depicted the adjuvants used or contained in the vaccine controls used in this Thesis. TLR (Toll-like receptor), Mincle (Macrophage inducible Ca^{2+} dependent lectin receptor), STING (Stimulator of Interferon genes), NLRP3 (NOD Like Receptor pyrin domain containing 3).

Adjuvants used in this Thesis

"Cationic Liposome Formulation" 01 (CAF®01)

CAF®01 is a liposome-based adjuvant that belongs to the wide family of "Cationic Liposome Formulation" (CAF) complex adjuvants, developed to enhance cell-mediated immune responses. CAF®01 contains two components: a quaternary ammonium surfactant N,N-dimethyl-N,N-dioctadecylammonium (DDA), and the synthetic mycolate α,α-trehalose 6,6′-dibehenate (TDB), an analog of the cord factor from *Mycobacterium tuberculosis*. Both components induce strong Th1-biased immune responses separately, but mixed together form stable liposomes that enhance their properties [131].

Glycolipids from mycobacteria like TDB are recognized by the C-type lectin receptor Mincle (Macrophage inducible Ca^{2+} dependent lectin receptor). Mincle is generally present in the surface of macrophages and dendritic cells for the detection of multiple ligands expressed in both bacteria and fungi. Mincle receptor binds carbohydrate residues that along to the coupling with Fcγ receptor, triggers a signalling cascade mediated by NF-κB, which drives to the production of proinflammatory cytokines and the dendritic cell activation (Figure 1.13) [132,133].

CAF®01 is a complex adjuvant and constitutes a delivery system as well, since encapsulates the antigen forming liposomes that form a transient depot at the injection site [134]. This depot allows the recruitment of migratory dendritic cells and facilitates the subsequent antigen intake and priming for further B and T cell induction in the draining lymph nodes, polarizing towards Th1/Th17 responses [135]. Although CAF

adjuvants were initially designed to develop a potent cell-mediated immune response, they are as well efficient in promoting humoral immunity in mice and ferrets [136]. The induced Th1 response helps skewing the IgG subclass switching towards IgG2 rather than IgG1, subclass more specialized containing high-affinity antibodies involved in opsonization and neutralization [137].

Depending on the nature of the antigen used for immunization, the immunogens can bind to the hydrophobic membrane core of the liposome or penetrate inside the lipid bilayer. These features may modify the retention and presentation of the antigen, affecting the subsequent antibody response [138]. These characteristics of CAF®01 adjuvant help overcoming the hyporeactiveness of neonate immunity by promoting Th1-type responses, since adaptive response in neonates is biased towards Th2 and Treg-type responses [139].

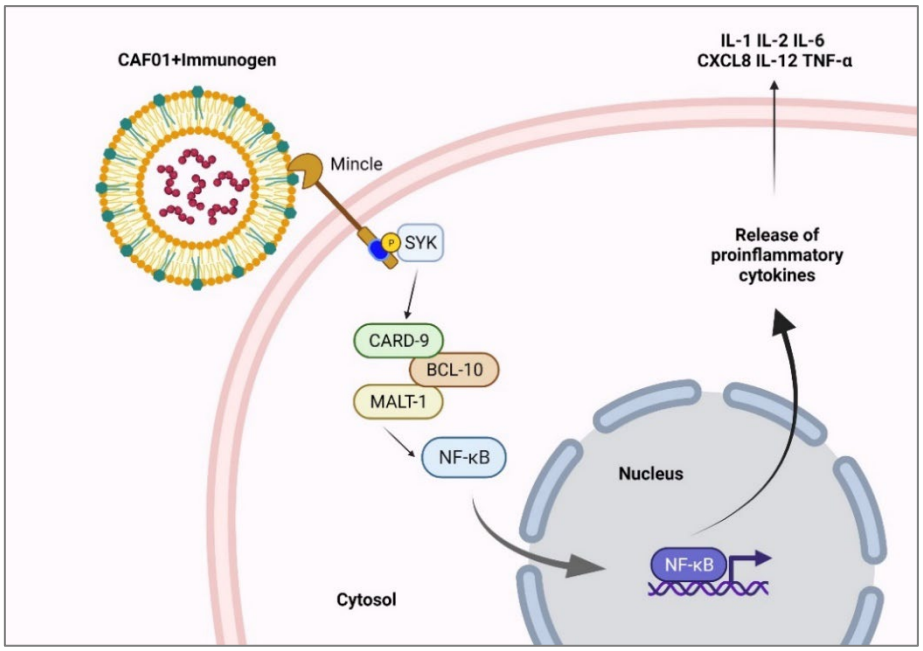

Figure 1.13. Mechanism of action of CAF®01 showing the signalling pathway triggered by the interaction of Mincle with its ligand that leads to the activation of NF-κB and the subsequent secretion of proinflammatory cytokines. Acronyms: Spleen associated tyrosine kinase (Syk), Caspase recruitment domain-containing protein 9 (CARD9), B-cell lymphoma/leukemia 10 protein (BCL-10), Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT-1), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Adapted from Pedersen et al. 2018 [131]. *Created with BioRender.com.*

Bis-(3′,5′)-cyclic dimeric adenosine monophosphate (CDA)

Bis-(3′,5′)-cyclic dimeric adenosine monophosphate (c-di-AMP or CDA) is a cyclic dinucleotide initially described in *Listeria monocytogenes* [140]. CDA belongs to a family of secondary messenger molecules involved in potassium and bacterial cell wall homeostasis function from bacteria and archaea [141].

Under natural conditions, dicyclic nucleotides and nucleic acid fragments are generated in the phagosome and released into the cytosol because of digested bacteria or processed virus in the phagocytic cells [142,143]. These nucleic acid fragments can activate the host innate immune system through the binding of different internal receptors located in the cytosol and the membrane of the endoplasmic reticulum. Cyclic dinucleotides are recognized by the stimulator of interferon genes (STING), which together with the ERAdP receptor, stimulates the secretion of interferons and proinflammatory cytokines through the NF-κB signalling pathway [144]. Similarly, the binding of CDA with inflammasome NLRP3 also stimulates the secretion of IL-1β in a STING independent manner (Figure 1.14).

CDA stimulates the expression of costimulatory molecules, maturation markers and cytokines in the dendritic cells that subsequently trigger a strong and balanced Th1/Th2/Th17-type responses with the secretion of proinflammatory cytokines, including interferons and IL-1β [145]. CDA also stimulates the proliferation of IFN-γ secreting cells, memory T and cytotoxic T cells (CTL) in experimental studies in mice [146]. Although CDA is an adjuvant designed for mucosal administration [147,148], it can also be applied by parenteral routes such as intramuscular or intradermal [149,150]. The application of CDA as an adjuvant has been studied extensively in the murine models and to a lesser extent in larger animal models.

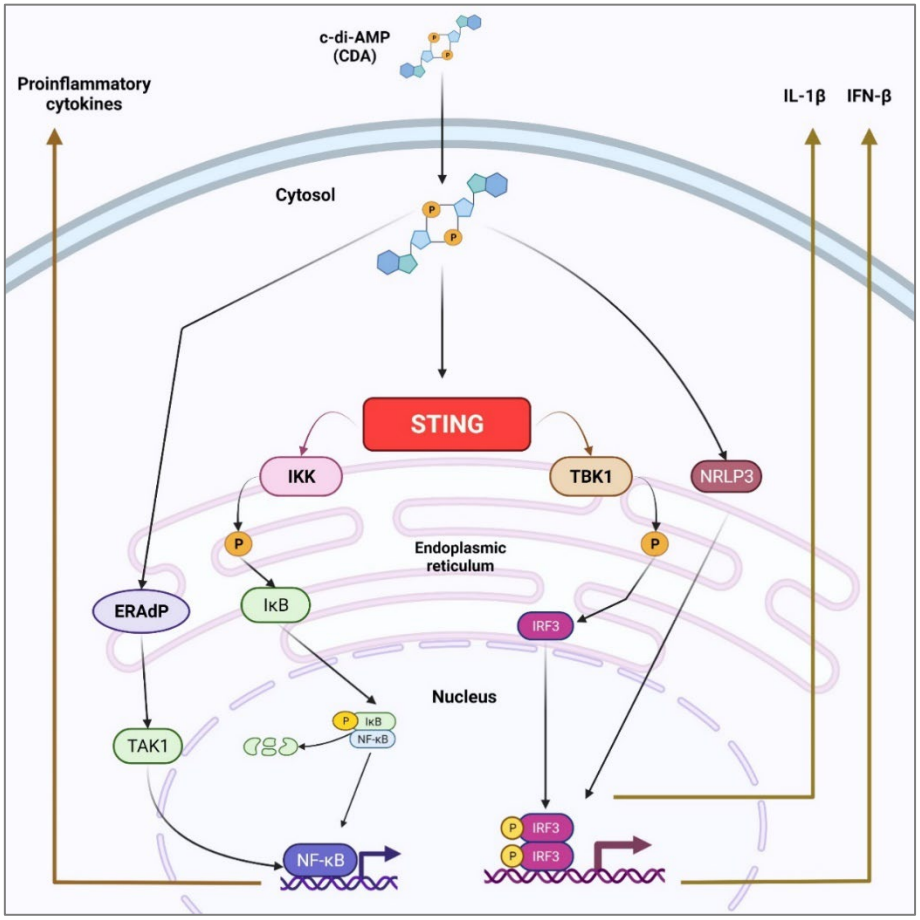

Figure 1.14. Simplified scheme of the mechanism of action exerted by CDA adjuvant. Modified from Yin et al. 2020 [141]. *Acronyms: Endoplasmic reticulum resident protein (ERAdP), Inhibitor of NF-kappaB kinase (IKK), Inhibitor of nuclear factor kappa B (IκB), Interferon Regulatory factor (IRF3), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), NLR family pyrin domain containing 3 (NLRP3), Stimulator of Interferon genes (STING), Transforming growth factor beta-activated kinase (TAK1), TANK-binding kinase 1 (TBK1). Created with BioRender.com.*

Other adjuvants included in this Thesis

α-Galactosyl ceramide methoxypolyethylene glycol

α-Galactosyl ceramide – methoxypolyethylene glycol (αGalCerMPEG or αGCM) is a pegylated form of the sphingolipid α-Galactosyl ceramide (αGC), a compound firstly isolated from the marine sponge *Agelas mauritanius* (Figure 1.15). αGC is an agonist of the MHC-I like molecule CD1d, a receptor of dendritic cells. The union of αGC together with CD1d is recognized by the TCRα chain of invariant Natural Killer T cells (iNKT). This union activates the cells and induces the rapid secretion of Th1/Th2 cytokines, leading to the stimulation of further immune cells of the adaptive response such as T helper subsets and CTLs [151]. αGC has been extendedly used as a mucosal adjuvant in several animal experiments against different viral diseases [151,152]. αGC is insoluble, making it difficult to combine with certain antigens, but this issue was solved by improving of its solubility with the addition of polyethene glycol [153].

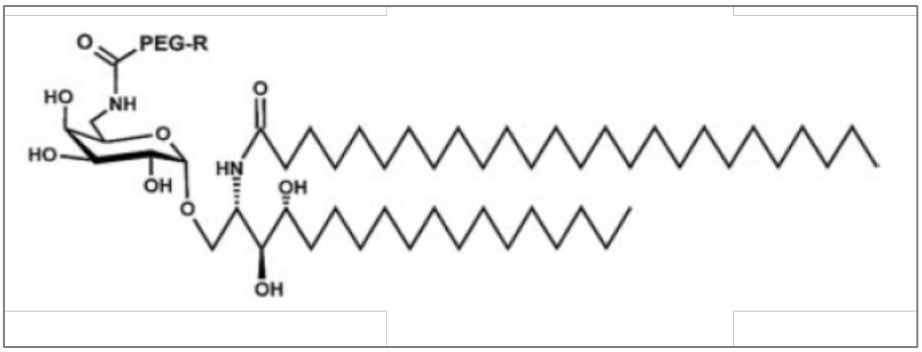

Figure 1.15. Molecular structure of α-Galactosyl ceramide methoxypolyethylene glycol (αGCM) adjuvant, from Ebensen et al 2007 [153].

Freund's Adjuvants

Freund's adjuvants are water-in-oil emulsions prepared from paraffin oil and surfactant mannide monooleate [154]. Developed in the 1930s by Jules T. Freund, there are two forms depending on their content: the complete Freund's adjuvant (CFA), which contains heat-killed *Mycobacterium tuberculosis* as an immunomodulator, and incomplete Freund's adjuvant (IFA), which is only formulated with the oil compounds [155]. Freund's adjuvants are some of the most used adjuvants in the research of autoimmune diseases and the induction of antibody production [156]. However, its use in human or veterinary medicine is banned due to the adverse side effects that include inflammation, pain, and distress. Indeed, the depot induced by Freund's adjuvant emulsions cause lesions at the injection site such as granulomatous inflammation [157]. The strong secretion of TNF-α and other proinflammatory cytokines attracts macrophages and dendritic cells to the inflamed tissue allowing an enhanced antigen presentation. Furthermore, the presence of the mycobacterial fraction ameliorates the adaptive immune response through the hyperactivation and proliferation of B and T cells in the draining lymph nodes leading to Th1 type response and the generation of high amounts of antibodies. Under experimental conditions, CFA is only used for the initial inoculations to avoid the generation of a specific immune response against the mycobacterial component that would counteract the antigen of interest. For this reason, boosters of Freund's adjuvant are carried out with the incomplete form (IFA) [156].

α-tocopheryl acetate

α-tocopheryl acetate is a synthetic form of vitamin E, an ester of acetic acid and α-tocopherol. It belongs to the family of tocopherol compounds, which possess adjuvant properties. α-tocopheryl acetate is nowadays exploited as an adjuvant in several veterinary vaccines for swine use (Diluvac® Forte). It is reported that the application of this adjuvant alone in vaccine formulations provides an increased humoral response as well as specific IFN-γ-mediated responses as observed against Aujeszky's disease virus [158]. α-tocopherol is also used currently in the emulsion-based adjuvant AS03 together with squalene and Tween 85, known for its strong monocyte and macrophage activation. Its inclusion in the AS03 adjuvant complex also provides a strong immune response with lower amounts of subunit antigen [159].

Specol® immunogenic adjuvant

Specol® is a water-in-oil emulsion containing the mineral oil Marcol 52 (composed of paraffins and cycloparaffins) beside the non-ionic surfactants Span 85 (sorbitan trioleate) and Tween 85 (polyoxyethylene sorbitan trioleate) [160]. Specol® is commercialized under Stimmune® brand and unlike CFA, lacks an immunomodulatory molecule. Once injected, the emulsion forms a depot in the tissue and produces an inflammation that facilitates the recruitment of immune cells. With the depot, the antigens can be released slowly, allowing a prolonged presentation, and avoiding their rapid diffusion into the bloodstream. Thus, APCs activation and migration to the draining lymph nodes

allows a further sustained stimulation and proliferation of T and B cells. Specol® offers an increased humoral immune response with relatively acceptable side effects and could be a good alternative to replace the use of CFA/IFA in vaccine formulations used in animal experiments [161,162]. Although the lesions caused at the injection site are milder compared to the ones produced by Freund's adjuvants, the depot can degenerate into a granuloma causing some discomfort and pain in the long term. For this reason, Specol® was only approved by the FDA for veterinary use.

Chapter II: Hypothesis and objectives

Chapter 2. Hypothesis and objectives

Hypothesis

An adequate immune response following vaccination is triggered by robust immunogenic antigens and guided by a fine coordination between the innate and adaptive immune systems. This coordination may lead to pathogen clearance and subsequent protection against infection. Conserved surface-proteins and peptide-derived antigens conform interesting strategies to obtain effective and universal (against all strains of a given pathogen) vaccines. However, these subunit antigens often lack sufficient immunogenicity and, therefore, they cannot provide an optimal immune response by themselves. Thus, the addition of adjuvants to subunit formulations would supply a robust immunity capable of responding against the infections, providing from efficacious and cross-protecting vaccines. Consequently, the addition of the adjuvants CAF®01 and CDA to vaccines consisting of subunit antigens, would enhance their immunogenicity and provide for new efficacious formulations for its use in pigs.

Objectives

Based on the hypothesis of this Thesis, the general objective was to define a good combination of antigen and adjuvant capable of reinforcing both the innate and adaptive immune response and confer protection. To this aim, purified antigens from porcine pathogens (virus, Gram-negative and Gram-positive bacteria) were combined with

CAF®01 or CDA adjuvants and tested in pigs. Thereby, to achieve this goal, the general objective was divided into three specific objectives:

1. To study the immune responses to vaccination with conserved hemagglutinin peptide NG34 from IV adjuvanted with CAF®01 or the combination CDA/αGalCerMPEG in young pigs and the subsequent challenge with a pandemic H1N1 IV isolate (**Chapter III**).

2. To evaluate the immune response (**Chapter IV**) and the protection capacity (**Chapter V)** of the conserved F4 protein fragment from the VtaA of virulent *G. parasuis*, adjuvanted with CAF®01 or CDA, in neonate piglets.

3. To determine the immunogenicity and efficacy of *S. suis* conserved antigens (MRP2 and C05) adjuvanted with CAF®01 or CDA in vaccinated neonate piglets (**Chapter VI).**

Chapter III. Study I: Immune Responses to Pandemic H1N1 Influenza Virus Infection in Pigs Vaccinated with a Conserved Hemagglutinin HA1 Peptide Adjuvanted with CAF®01 or CDA/αGalCerMPEG.

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Chapter 3. Study I: Immune Responses to Pandemic H1N1 Influenza Virus Infection in Pigs Vaccinated with a Conserved Hemagglutinin HA1 Peptide Adjuvanted with CAF®01 or CDA/αGalCerMPEG

Abstract

This study aimed to evaluate the immune response and protection correlates against Influenza virus (IV) infection in pigs vaccinated with the novel NG34 HA1 vaccine candidate ad-juvanted with either CAF®01 or CDA/αGalCerMPEG (αGCM). Two groups of six pigs each were vaccinated intramuscularly twice with either NG34+CAF®01 or NG34+CDA/αGCM. As controls, groups of animals (n = 6 or 4) either non-vaccinated or vaccinated with human seasonal trivalent influenza vaccine or NG34+Freund's adjuvant were included in the study. All animal groups were challenged with the 2009 pandemic (pdm09) strain of H1N1 (total amount of 7 × 106 TCID50/mL) via intranasal and endotracheal routes 21 days after second vaccination. Reduced consolidated lung lesions were observed both on days three and seven post-challenge in the animals vaccinated with NG34+CAF®01, whereas higher variability with relatively more severe lesions in pigs of the NG34+CDA/αGCM group on day three post-infection. Among groups, animals vaccinated with NG34+CDA/αGCM showed higher viral loads in the lung at seven days post infection whereas animals from NG34+CAF®01 completely abolished virus from the lower respiratory tract. Similarly, higher IFNγ secretion and stronger IgG responses against the NG34 peptide in sera was observed in animals from the

NG34+CAF®01 group as compared to the NG34+CDA/αGCM. NG34-vaccinated pigs with adjuvanted CAF®01 or CDA/αGCM combinations resulted in different immune responses as well as outcomes in pathology and viral shedding.

Introduction

Influenza is a contagious disease caused by Influenza viruses (IV) that mainly can affect birds, which represent the natural reservoir and mammals that act as natural hosts [40]. Within mammals, IV can cause disease in a wide range of groups including carnivores, human and pigs [163]. Although only three subtypes of Influenza A viruses (IAV) make up the vast majority of influenza infections in pigs worldwide (H1N1, H1N2 and H3N2) [42], the high variability between strains makes the production of efficacious vaccines for the prevention and control of the disease a big challenge to vaccine-manufacturers. Swine influenza viruses (SwIV) not only cause significant economic losses for the swine industry, but also are important zoonotic pathogens since variant viruses in swine pose threat for humans, e.g., H1N1 2009 pandemics. Moreover, swine represent a model of choice for the research of Influenza infection and immunity among other animals like mice and ferrets [164].

Vaccination is considered the most important and effective strategy to prevent and control IAV infection and disease in both animals and humans. Current strategies to combat IAV infection include vaccines that consist seasonal trivalent/quadrivalent Influenza Virus (IV) strains,

based on inactivated virus or its corresponding hemagglutinins with or without additional adjuvants [165]. The immune responses triggered by these vaccines; however, these are strain specific and do not protect individuals against heterologous emerging strains, because of the characteristic mutating nature of IVs. Multivalent or universal vaccines, based on conserved antigen motifs from influenza virus, could be an attractive albeit challenging strategy to broadly prevent influenza virus infection and reduce the risk of influenza pandemics [166–168]. Conserved antigen subunits, on the other hand, are often poor immunogens and may require additional adjuvants to induce the strong humoral and cellular immune responses needed to overcome IV infection [169].

Our research group has extensively worked to identify and select biologically active antigen subunits from Hemagglutinin 1 (HA1) of IAV for the development of a universal vaccine. Using an informational spectrum method (ISM) [170], a 34 amino acid antigen subunit (NG34) from HA1 of IAV was selected as a potential vaccine candidate. NG34 is located within the immunogenic site E in the N terminus of HA1, a domain close to receptor binding site of the HA characterized as well conserved. Recently, we have demonstrated that immunization with the NG34 antigen either incorporated in a plasmid or as a peptide formulated with adjuvants like Montanide®, Diluvac® Forte, Addavax® or Alhydrogel induced specific antibodies as well as CD4 T cell responses, that conferred protection against homologous and heterologous IV infection in a pig model. The robust characteristic of

this immunogenic NG34 peptide has been demonstrated in different experimental animal infection models [92,97].

In the present study we examined the immune correlates that may define protection against IV infection in pigs immunized with the NG34 peptide adjuvanted with either a liposome based "Cationic Adjuvant Formulation 01" (CAF®01) or a combination of bis-(3,5)-cyclic dimeric adenosine monophosphate (CDA) and α-galactosylceramide methoxypolyethylene glycol (αGCM). The adjuvant CAF®01 is composed of ammonium surfactant N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) and C-type lectin receptor (Mincle) agonist α,α'-trehalose 6,6'-dibehenate (TDB) a syntethic glycolipid analog to the cord factor from *Mycobacterium tuberculosis*. CAF®01 is known to induce Th1/Th17 type cell mediated immunity as well as strong humoral responses [131]. It has furthermore been shown to effectively improve TIV efficacy both against homologous and heterologous IAV infection [136,171]. CDA is a monocyclic dinucleotide secreted by *Listeria monocytogenes* that is known to activate the "Stimulator of Interferon Genes" (STING) in the host, leading to the activation of TNF and type I IFN that stimulate Th1/Th2/Th17 and cytotoxic cellular and humoral immune responses [146]. The αGCM is a pegylated glycolipid derived from the marine sponge *Agelas mauritianus* and is a superagonist for iNKT cells involved in immune-modulation, stimulation of the Th2 response and enhancement of mucosal antibody response [153]. In addition to the mentioned adjuvants, two groups of pigs were treated, respectively, with Seasonal

Trivalent Influenza Vaccine (TIV) and NG34 + Freund's Adjuvants used as historical controls to evaluate their effectivity and immunogenicity.

Materials and Methods

Vaccine Antigens and Adjuvants

NG34 peptide, which sequence corresponds with 34 amino acids located in the E site from the N terminus of HA1 from A/Catalonia/63/2009 strain (pdm09 H1N1, positions 87 to 120 [GenBank ACS36215], was synthesized by CASLO ApS (Kongens Lyngby, Denmark). The lyophilized NG34 was reconstituted to a concentration of 2 mg/mL in ammonium chloride solution following manufacturer's instructions and stored at −80°C until use. Integrity of the peptide sequence was confirmed before the experiment by MALDI-TOF mass spectrometry.

CAF®01 and CDA/αGCM adjuvants were provided by the collaborator laboratories of this study, the *Statens Serum Institut* (Copenhagen, Denmark) and *Helmholtz-Zentrum für Infektionsforschung* (Braunschweig, Germany), respectively. Vaccine formulations were prepared according to its instructions in the recommended proportions (Table 3.1). Complete (CFA) and incomplete (IFA) Freund's Adjuvant were purchased from Sigma Aldrich (Madrid, Spain). Animals primed with CFA and boosted with IFA adjuvant in combination with NG34 were considered as a positive control group for the adjuvants.

Table 3.1. Distribution of experimental groups and composition of the different antigen and adjuvant combinations.

Experimental Group	N	Antigen	Adjuvant
1. Unvaccinated/unchallenged (PBS-NV/NC)	6	PBS	None
2. Unvaccinated/challenged (PBS-NV/C)	6	PBS	None
3. NG34–CAF®01	6	50 μg of NG34	980 μL of CAF®01
4. NG34–CDA/αGalCerMPEG (CDA/αGCM)	6	50 μg of NG34 *	25 μg of CDA + 25 μg αGCM
5. NG34–Freund's Adjuvant (FA)	4	50 μg of NG34 *	600 μL of CFA/IFA[1]
6. Seasonal Trivalent Influenza Vaccine (STIV)	6	500 μL of Chiroflu® 2018–19 seasonal vaccine[2]	

One mL of each vaccine formulation per animal was injected except for STIV, where the dose consisted of 0.5 mL. *: Vaccine antigen diluted in PBS. 1: First immunization was performed using Complete Freund's Adjuvant (CFA); booster was prepared using Incomplete Freund's Adjuvant (IFA). 2: Including hemagglutinins from: A/Singapore/GP1908/2015 (similar strain to A/Michigan/45/2015 (H1N1) pdm09); A/Singapore/INFIMH-16-0019/2016 (H3N2) and B/Maryland/15/2016 wild type (similar strain to B/Colorado/06/2017).

Unadjuvanted human TIV vaccine used in the Portugal and Spain influenza vaccination campaign of 2018–2019 Chiroflu® (Seqirus Srl., Siena, Italy) was used in this study to benchmark against an approved vaccine. Its composition comprised 15 μg of the three hemagglutinins from the following strains produced in egg: A/Singapore/GP1908/2015 (similar strain to A/Michigan/45/2015 (H1N1) pdm09); A/Singapore/INFIMH-16-0019/2016 (H3N2) and B/Maryland/15/2016 wild type (similar strain to B/Colorado/06/2017).

Cell Cultures and Virus

Madin-Darby Canine Kidney cells (MDCK, ATCC CCL-34) were used for virus propagation, titration and seroneutralization assays; cells were cultured in Dulbecco's Modified Eagle Medium DMEM (Lonza, Basel, Switzerland) supplemented with 5% of fetal bovine serum (Euroclone, Milan, Italy), 1% of L-glutamine (Gibco Life Technologies, Madrid, Spain) and 1% penicillin-streptomycin (Gibco Life Technologies, Madrid, Spain) at 37°C 5% CO_2.

A/Human/Catalonia/63/2009 pandemic (pdm09) H1N1 Influenza virus available in the laboratory was propagated in MDCK cells. Briefly, monolayer cell cultures were inoculated with the help of 10 μg/mL porcine trypsin (Sigma-Aldrich, Madrid, Spain) at a MOI of 0.0001 to obtain the desired concentration at the harvest two days later. Subsequently, cultures were frozen to rupture infected cells and centrifuged. Supernatants were stored until use at −80°C. Titration of inocula were performed by culture of serial dilutions in MDCK cells and the resulting $TCID_{50}$/mL was calculated using the Reed and Muench method [172].

Some volume of viral production was UV light inactivated under a lamp to use for cell stimulation. Briefly, 1 mL volume of viral stock was dispensed in a six-well cell culture plate to reach a 1 mm of thickness under the UV light for 20 min. This procedure was repeated until the desired volume was inactivated. Once obtained, viral bulk was tested for viability by serial dilution cultures and read by cytopathic effect as

it is described above. Inactivated viral volumes were aliquoted and frozen at −80°C until use.

Ethics regulation

This experiment was performed at BSL3 animal house facilities located in IRTA-CReSA (Bellaterra, Catalonia, Spain). This study was conducted according to the ARRIVE and the Declaration of Helsinki guidelines approved by IRTA's Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Catalonia Government (Spain) with number 133/2019 in compliance with the Directive, EU 63/2010, the Spanish Legislation (RD 53/2013) and the Catalan Law 5/1995 and Decree 214/1997.

Experimental Design

Clinically healthy Landrace x Large White pigs from livestock farms (Selecció Batallé, Riudarenes, Girona, Spain) of about six weeks-of-age and similar weight (10–13 kg) were firstly screened for Influenza NP protein antibodies by ID Screen® Influenza A Antibody Competition ELISA (IDVET, Grabels-Montpellier, France). Thirty-four seronegative animals were selected and then distributed randomly into six experimental groups of six or four pigs blocking by obtained ELISA titre (Table 3.1). All groups were split in two boxes at BSL3 facilities of IRTA-CReSA with three animals per group in each box; therefore, all groups were represented within the same box, sharing the air space. Prior to the experiment all animals were confirmed negative for IV twice (at the

selection and before the beginning of the experiment) using RT-qPCR [173] (see below, RT-qPCR–Viral load section) to ensure they were not exposed to IVs.

Animals were immunized on study days 0 and 21; 39 days after the first vaccination, animals were challenged with pdm09 H1N1 IV strain by two routes: intranasally using a nebulizer with 1 mL of 106 TCID50/mL per nostril and by endotracheal route inoculating 5 mL of 106 TCID50/mL (Figure 3.1). Uninfected animals were inoculated in both ways with sole viral propagation medium (DMEM 1% Gln 1% penicillin-streptomycin). Half of the animals per group were sacrificed on three days post-inoculation (dpi) and the remaining animals on day seven post-inoculation (p.i.). Euthanasia of the animals was performed by an intravenous overdose of sodium pentobarbital (140 mg/kg). During all experimental procedures, animals were fed ad libitum and were not treated with antibiotics, anesthetics, or analgesics since they were not suffering from any clinical condition that required such intervention.

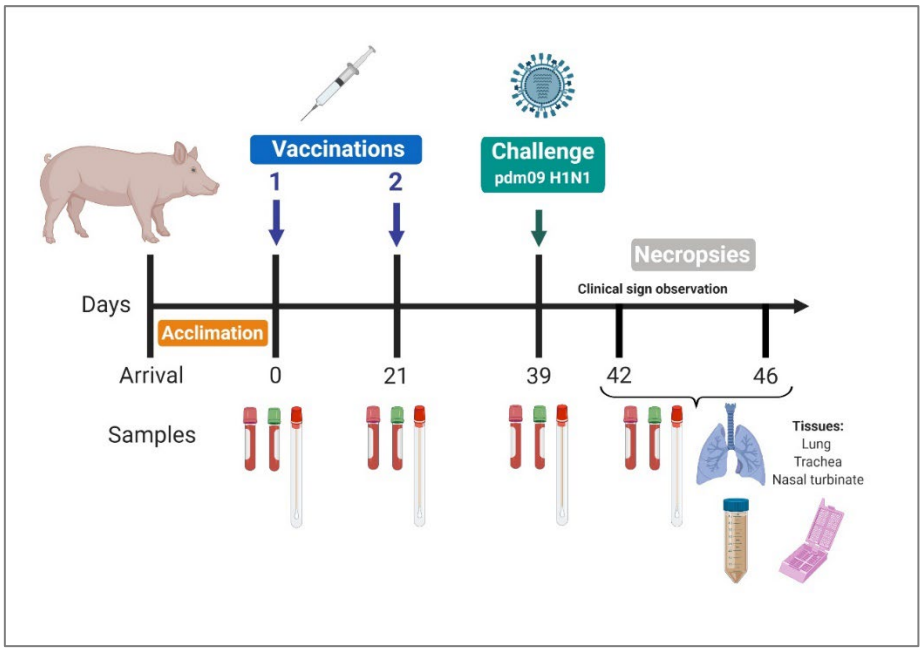


Figure 3.1. Schematic representation of the vaccination study. Thirty-four young piglets of 6 weeks-of age were distributed into 6 groups in the experimental facilities and left one week for acclimation. Animals were twice immunized in an interval of 21 days; on day 39 they were challenged by intranasal and endotracheal routes with 10^7 $TCID_{50}$ of pdm H1N1 A/Human/Catalonia/63/2009. After challenge, half of the animals were euthanized on day 3 (D42) and 7 (D46) post infection. Samples of nasal swabs and blood were collected in the designated timepoints as well as samples of BALF and tissues in the necropsy days. Figure created with BioRender.com.

Sampling

Samples were collected at vaccination, challenge, and necropsy, comprising two nasal swabs, clotted blood for sera and EDTA-treated whole blood for PBMCs. Blood was collected from the jugular vein with Vacutainer tubes (Becton Dickinson, CA, USA); sera were obtained by centrifuging the tubes 10 min at 2500 rpm (1258 g) at room temperature. Two nasal swabs collected from both nostrils were resuspended in 1 mL

of PBS, supplemented with 1% penicillin-streptomycin (Gibco Fisher Scientific, Waltham, MA, USA). Post-inoculation, nasal swabs were collected daily until the end of the experiment. In addition, necropsy samples comprised portions of lung (apical, medial, and cranial part of diaphragmatic left lobes), trachea and nasal turbinates for histopathological assessment conserved in 10% formalin. Bronchoalveolar lavage (BALF) was obtained dissecting the right lung and filling it with 150 mL of sterile PBS, recovering after a smooth massage a final volume of about 50 mL of lavage. Serum samples, nasal swabs and BALF were stored at −80°C until use.

Clinical Signs and Pathological Assessment

Rectal temperatures and flu-like clinical signs were evaluated throughout the whole experimental period. Fever was considered when rectal temperature values were above 40°C. To assess gross lesions caused by infection, individual lungs were collected on necropsy days and pictures of dorsal and ventral sides were taken. The macroscopic affected area was quantified by image analysis (ImageJ online free software), a scoring system was applied as previously described [174].

Formalin fixed tissues were embedded in paraffin wax and sectioned 3–5 μm to stain with hematoxylin-eosin for histopathologic assessment and with monoclonal antibody from hybridoma ATCC No. HB-65 against AIV nucleoprotein for immunohistochemistry as it is described before [175].

Quantitative PCR RT-qPCR–Viral Load

Nasal shedding and viral load were assessed through quantitative RT-PCR for M protein as it has been previously described [173]. Viral RNA was extracted from resuspended nasal swabs and BALF using IndiMag Pathogen Kit (Indical Bioscience, Leipzig, Germany) following the manufacturer's instructions. Subsequently, the TaqMan RT-qPCR mentioned before was run in Fast7500 Thermocycler equipment (Applied Biosystems, Foster City, CA, USA).

Samples with undetectable fluorescence were considered negative. Genome equivalent copies were calculated per sample using a standard curve. An arbitrary Ct value of 39.5 (below the detection level of the technique) was given to those negative samples for statistical analyses. Area under the curve (AUC) of the nasal shedding was determined using AUC function from Prism v6 (GraphPad Software, San Diego, CA, USA). AUC of each animal was calculated until 3 dpi and 6 dpi respectively; afterwards, mean and SD were calculated for each group.

Assessment of IFNγ Producing Cells

In order to evaluate secretion of IFNγ under different stimulations, an IFNγ Enzyme-Linked ImmunoSpot Assay (ELISPOT) was performed. Peripheral blood mononuclear cells PBMCs were isolated from 10 mL of EDTA-treated blood from all animals at different timepoints (0, 21, 39 days; 3 and 7 dpi). Cell isolation was done by a density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, Madrid, Spain),

followed by an osmotic shock to remove the red blood cells. PBMCs were adjusted to 5×10^5 cells/well and plated in cell culture plates with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, 1% Glutamine, 1% penicillin-streptomycin and β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain). Cells were incubated for 18 h at 37°C 5% CO2 in precoated high binding 96-well plates (Costar Corning Incorporated, New York, NY, USA) with porcine IFNγ antibody (BD Pharmingen™, San José, CA, USA) in the presence of the following stimulus: Phytohemagglutinin (Sigma-Aldrich, Madrid, Spain) as positive control, recombinant Hemagglutinin 1 from A/Human/California/001/2009 strain (Sino Biologicals, Eschborn, Germany), UV inactivated virus A/Human/Catalonia/63/2009 and NG34 peptide. After incubation, plates were then washed to remove attached cells and stained with biotylinated IFNγ antibody (BD Pharmingen™, San José, CA, USA) and streptavidin (Invitrogen Life technologies. Madrid, Spain), followed by a development with insoluble TMB (Merck Life Science, Madrid, Spain). Resulting spots were counted under the Stereoscopic Zoom Microscope SMZ800 (Nikon Instruments Inc., Chiyoda, Japan).

Humoral Immune Response Evaluation

Humoral response was analyzed through an in house ELISAs against HA1 from A/Human/California/001/2009 and NG34 peptide in sera and BALF for total IgG, IgG1, IgG2 and IgA. High binding 96 well plates

(Costar Corning Incorporated, New York, NY, USA) were coated with the analyte of interest in carbonate buffer and incubated overnight at 4°C. After blocking the plates, samples were incubated 1 h at 37°C and later stained with rabbit anti-pig IgG H+L HRP conjugated (Sigma-Aldrich, Madrid, Spain) to detect total IgG. With BALF samples, same antibodies including goat anti-pig IgA HRP conjugated (AbDSerotec, Oxford, UK) for IgA were included. IgG isotypes in sera were assessed staining the samples with mouse anti-pig IgG1 or mouse anti-pig IgG2 antibodies (Bio-Rad Laboratories, Madrid, Spain) followed by a staining step with goat anti-mouse IgG HRP conjugated (Sigma-Aldrich, MO, USA). Staining steps with antibodies were carried out for 1 h at 37°C; after washes, plates were developed with soluble TMB (Sigma-Aldrich, MO, USA) and stopped with 1N H_2SO_4. Plates were read in a Power Wave XS spectrophotometer (Biotek Instruments, Winooski, VT, USA) at 450 nm wavelength. Swine IV HA1-positive and negative sera (GD Animal Health, Deventer, The Netherlands) and NG34-positive serum were included as internal controls for the technique. Thresholds of positive values were considered above the mean of the negative animals plus three times their standard deviation.

Hemagglutination Inhibition and Neutralization Assays

To assess the level of protecting antibodies, two different assays were performed: hemagglutination inhibition (HI) assay and neutralization (NT) assay in BALF with MDCK cells. For both procedures, protocols from WHO [176] and OIE (WOAH) [177] were followed and are briefly

described below. Challenge strain A/Catalonia/63/2009 pdmH1N1 was used for both techniques as well as reference sera from GD Animal Health (Deventer, The Netherlands) were included as positive and negative controls.

A total of 5 mL of fresh blood was obtained from 3 chicken by cardiac puncture and mixed with Alsever's solution (1:1). Red blood cells were washed twice with PBS centrifuging at 1115 rpm (250 g) for 10 min and adjusted with PBS to a final concentration of 50% for hemadsorption and 0.5% for hemagglutination and inhibition assays.

Sera from all sampling timepoints were treated with RDE II Seiken (Denka Chemicals, Tokyo, Japan) for 18 h at 37°C, followed by a heat-inactivation for 1 h at 56°C and subsequent hemadsorption. Sera were two-fold diluted in PBS in a v-bottomed 96 well plate; 25 μL of viral antigen diluted to 4 Hemagglutination Units (HAU) was dispensed to each well and incubated for 1 h at room temperature. After that, 25 μL of 0.5% of red blood cells were added to the mixture; after 1 h, plates were tilted to evaluate hemagglutination. Antibody titres were considered as the reciprocal dilution where the inhibition was complete; seroprotective titres were considered above 1/40.

For BALF neutralization (NT) assay, samples were heat inactivated at 56 °C for one hour and two-fold diluted in DMEM 1%Gln 1% P-ST and mixed with challenge virus with 100 $TCID_{50}$/well for two hours at 37°C at 5% CO_2. After incubation, a post-infection medium (DMEM 1%Gln 1% P-ST) with the help of 10 μg/mL porcine trypsin (Sigma-Aldrich, Madrid, Spain) was added to plates and incubated for one week until

examination for cytopathic effect. Titres were expressed as the reciprocal dilution where no cytopathic effect appeared.

Statistical Analysis

Graphs and statistical analysis were performed using Prism v6 (GraphPad Software, San Diego, CA, USA). Raw data was ln(log) transformed to reach gaussian distribution and confirmed using the Shapiro–Wilk test. Statistical differences were analyzed by ANOVA. Afterwards, post-hoc multiple comparisons between vaccinated groups and NV/C group were performed using Dunnett's test. Statistical significance was denoted as it follows in each graph: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Results

Vaccination with NG34+CAF®1 reduced pulmonary lesions after challenge with pdmH1N1

After the inoculation with pdm09 IAV, pigs did not display evident respiratory clinical signs. However, they developed a peak of pyrexia (rectal temperature >40°C) and lethargy one day after the infection, without significant differences between groups (Figure 3.2A). This pyrexia was resolved two dpi and temperatures remained constant during the following days, where rectal temperatures remained below 40 °C until the termination of the study in all the animals. Dyspnoea, coughing, abnormal breathing, nasal/ocular discharge, or conjunctivitis

were not observed during all the experimental procedure. No clinical signs or fever was observed in non-infected control pigs.

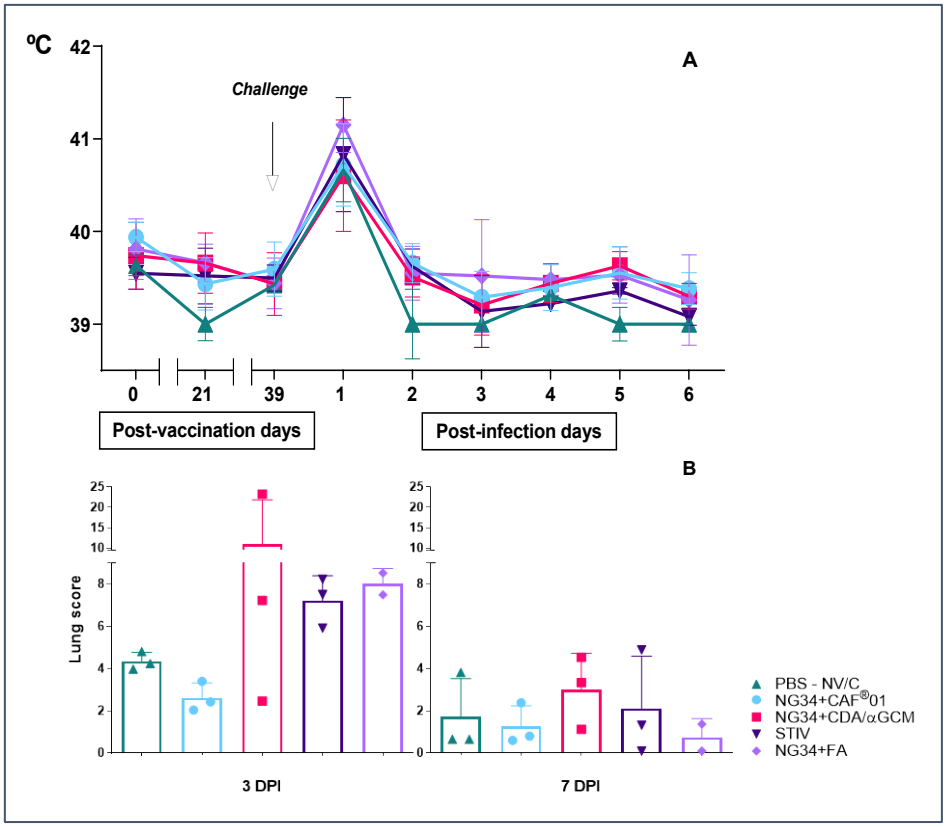


Figure 3.2. Graphs depicting average and SD of rectal temperatures taken during the experiment (A) and Influenza like pulmonary cranio-ventral consolidation lesions observed in animals euthanized at 3 and 7 days after the infection (B).

At necropsy days, gross lesions characterized by pulmonary cranio-ventral multifocal consolidation were observed in lungs of inoculated animals. Numerically higher scores of typical flu-like areas were observed in animals euthanized at three dpi compared to animals

sacrificed at seven dpi (Figure 3.2B). Moreover, broncho-interstitial pneumonia compatible with IV infection was confirmed in all inoculated animals at microscopic level but with no significant differences between them. Compared to the non-vaccinated group, pulmonary scores of the group vaccinated with NG34+CDA/αGCM were highly variable on three dpi, having one animal with extremely severe lesions and another one where lung lesions were almost negligible. Although reduced by seven dpi, variability within the animals vaccinated with NG34+CDA/αGCM remained higher in comparison to other vaccinated and challenged animal groups. On the contrary, IAV associated lung lesions in animals vaccinated with NG34+CAF®01 were relatively homogeneous and considerably less severe in all animals both on three and seven dpi compared to pigs from the non-vaccinated group (Figure 3.2B). Animals vaccinated with TIV showed a high lesion score on three dpi that were greatly reduced on seven dpi. Difference between both timepoints was detected ($p<0.001$). However, all the described variability and differences between groups were not statistically significant in both timepoints ($p>0.1$). Tables with pictures of ventral and dorsal sides of lungs from infected animals are available in the appendix (Supplementary table 1-4).

Viral shedding varied between experimental groups

RT-qPCR was used to explore nasal viral shedding and viral load in BALF from studied animals. All groups exhibited similar pattern of nasal virus load having a peak 4 dpi with virus titres in relative numbers

around Log_{10} 5 GEC (genome equivalent copies). Nonetheless, 2 out of 3 animals of the group vaccinated with NG34+CAF®01 had undetectable viral genome levels at four dpi. This animal group also showed relatively lower virus secretion during the whole experimental infection period, except for one animal that presented high viral load in nasal swabs on day 4 and 5 post-challenge. The other groups, vaccinated with seasonal TIV or NG34 in combination with CDA/αGCM or Freund's adjuvants, showed a decreasing trend in nasal viral load from day five onwards compared to control non-vaccinated challenged animals. None of these results were statistically significant ($p>0.1$) (Figure 3.3A).

Regarding AUC calculation, all vaccinated groups had a lower value than the NV/C group at 3 dpi, being the TIV group the one with the lowest AUC. Same effect was observed at 6 dpi, when the NG34+FA group presented the lowest AUC. However, these results were not significantly different ($p>0.1$) among groups (Table 3.2).

Table 3.2. Area under the curve calculation of the nasal shedding performed until 3 dpi and 6 dpi.

Experimental Group	AUC Until 3 dpi (n = 6)		AUC Until 6 dpi (n = 3)	
	Mean	SD	Mean	SD
PBS-NV/C	0.6052	±0.6680	4.1520	±0.9719
NG34 + CAF®01	0.2397	±0.2316	2.2317	±2.2538
NG34 + CDA/αGCM	0.6228	±0.6968	2.8888	±2.3776
STIV	0.1255	±0.1180	2.5247	±0.4745
NG34 + FA*	0.3036	±0.3506	1.4695	±2.0782

* NG34 + FA group: AUC calculated until 3 dpi (n = 4), 6 dpi (n = 2).

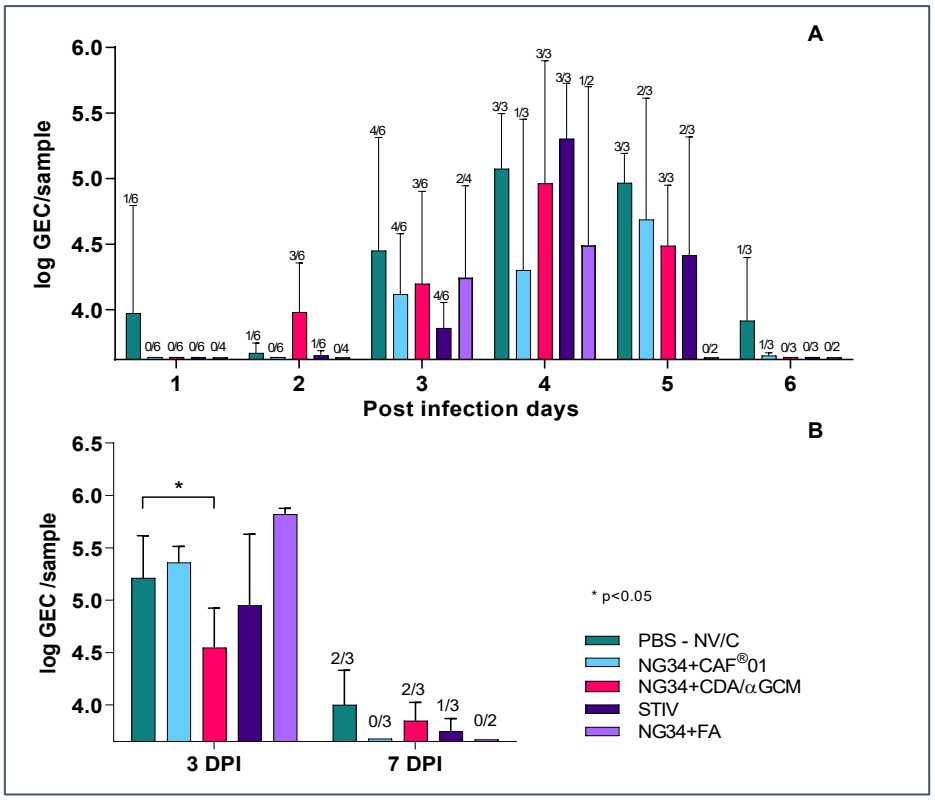


Figure 3.3. Results obtained by RTqPCR analysis from nasal swabs (A) and bronchoalveolar lavage (B). Viral load in nasal swabs is represented as log GEC/sample of Influenza M protein gene from purified RNA of nasal swabs or BALF. Bars express averages and error bars the SD of each group. The number of animals with positive signal in qRT-PCR are represented above each bar in the figure.

Virus load in the BALF from animals sacrificed on 3 dpi was quite homogenous in all vaccinated infected animals and no significant differences between vaccinated or control non-vaccinated infected animal groups was detected, with the exception of animals receiving NG34+CDA/αGCM that showed significantly reduced ($p<0.05$) viral loads (Figure 3.3B). A noteworthy difference was observed on day 7 p.i. where the virus load was below detection levels in all pigs from the

NG34+CAF®01 vaccinated group (two-way ANOVA $p<0.0001$; Dunnett's test $p<0.05$). These results were further confirmed by analyzing virus load in the lung tissues homogenates taken on days 3 and 7 post-challenge. Where a residual presence of viral genome was detected albeit GEC were extremely low at day 7 after infection in all animal groups (data not shown).

PBMCs from NG34+CAF®01 vaccinated animals secreted IFN-γ upon the stimulation with NG34

Vaccine induced IV specific-T cell response was evaluated by measuring IFNγ producing cells by ELISPOT. PBMCs were harvested at defined time points before and after challenge from non-vaccinated and vaccinated (NG34+CAF®01, NG34+CDA/αGCM, NG34+FA and TIV) animals and were subjected to different stimulus (UV inactivated pdm09 H1N1, HA1 or NG34) in vitro. The number of IFNγ producing cells varied depending on the stimulus used.

PBMCs isolated from all vaccinated animal groups showed an increase in the number of IFNγ producing cells stimulated in vitro with inactivated pdm09 H1N1 virus (Figure 3.4A), HA1 (Figure 3.4B) and NG34 (Figure 3.4C) on day seven post-challenge. These increments were statistically significant only in NG34+CDA/αGCM and STIV groups ($p<0.0001$) compared to non-vaccinated, challenge control animal group. Similarly, PBMCs stimulated in vitro with purified HA1 protein from pdm09 H1N1 only reacted against the antigen 7 days after

the infection with significant differences with respect the non-vaccinated animals in NG34+CDA/αGCM, NG34+FA and TIV groups.

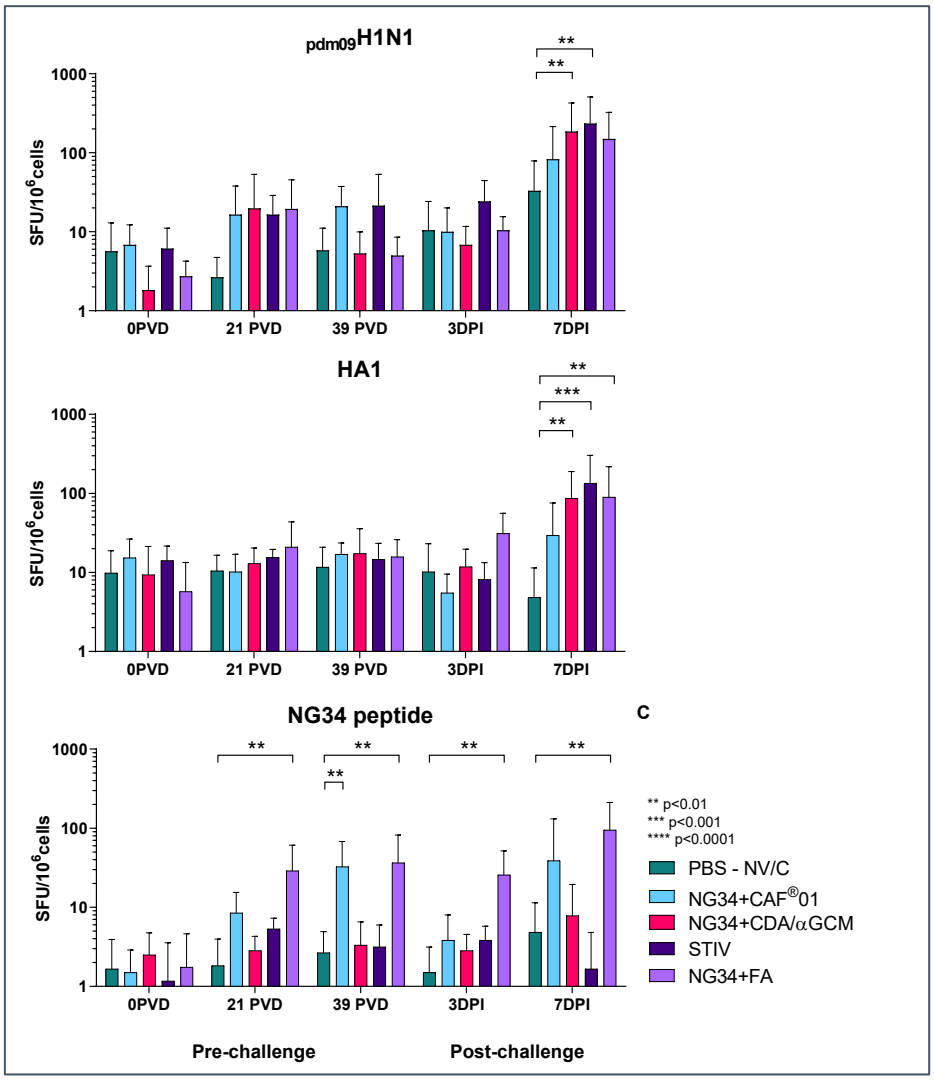


Figure 3.4. IFNγ ELISPOT results obtained in stimulated PBMCs from blood collected during the study with UV inactivated pdm09 H1N1 A/Human/Catalonia/63/2009 (A); recombinant HA1 (B) and NG34 peptide (C).

Under NG34 peptide stimulation, the animals vaccinated with NG34+FA reacted progressively throughout the different vaccination and after challenge timepoints and becoming significant again ($p<0.01$), as the rest of the stimulus on day 7 after infection. Moreover, only groups vaccinated with NG34+CAF®01 or Freund's adjuvant reacted against the antigen and with notable difference in comparison with the first timepoint, although for NG34+CAF®01 the differences were only statistically significant before the challenge (39 PVD).

CAF®01 elicited enhanced immune response than CDA/αGCM in combination with NG34

Antibody response was analyzed against the NG34 epitope and the complete HA from the pdm09 H1N1 IAV (Figure 3.5). A significantly increased NG34-specific IgG response, however, was noted in animal groups vaccinated with NG34+CAF®01 similar to NG34+FA. This response remained elevated after challenge and displaying statistically significant differences ($p<0.0001$) compared to the non-vaccinated challenged group (Figure 3.5A). The NG34+CDA/αGCM vaccinated group showed a weak IgG response against NG34 with only one animal responding higher than background levels. None of the remaining vaccinated groups showed NG34-specific IgG response. This trend remained similar after the challenge where significantly higher IgG response against NG34 antigen was only observed in animals vaccinated with NG34+CAF®01 and NG34+FA (Figure 3.5A). Further analysis of antibody isotype revealed that both the NG34-specific IgG1

and IgG2 were significantly elevated in pigs from the animal group vaccinated with NG34+CAF®01 and NG34+FA (Figure 3.5B and C). The rest of the vaccinated groups (NG34+CDA/αGCM, STIV) were barely inducing IgG1 or IgG2 titres. Systemic HA1-specific IgG titres were only observed in animal group vaccinated with TIV after challenge with pdm09 H1N1 (data not shown).

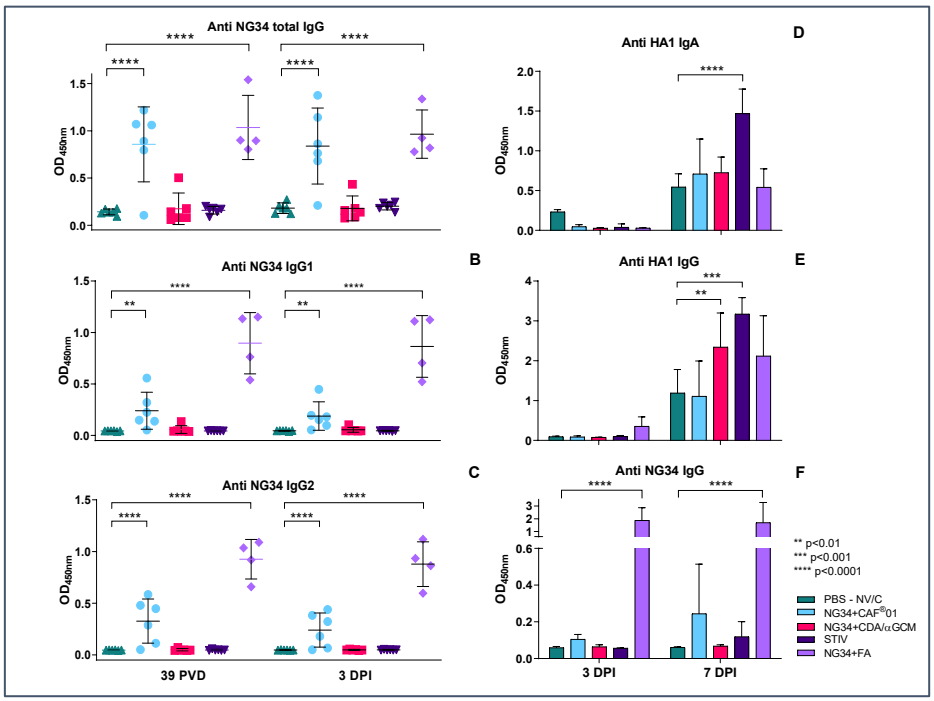


Figure 3.5. Antibody response in sera and BALF. Total Anti NG34 IgG (A), Anti Ng34 IgG1 (B), Anti NG34 IgG2 (C), Anti HA1 IgG in BALF (D), Anti HA1 IgA in BALF (E) and Anti NG34 IgG (F).

Presence of antibodies in BALF at necropsy time points was also evaluated. HA1-specific IgA and IgG in BALF in all animal groups were observed only 7 days after challenge (Figure 3.5D and E). HA1-specific IgG and IgA response in BALF was significantly higher in the group

vaccinated with TIV ($p<0.001$) than the rest of vaccinated animals. Vaccination with NG34+CDA/αGCM also induced a statistically significant HA1-specific IgG response in BALF ($p<0.01$), but no IgA response could be detected in the BALF collected from animals vaccinated with NG34+CDA/αGCM (Figure 3.5E). None of the vaccinated groups except NG34+FA showed specific NG34-IgG response on day 3 after challenge in the BALF. A positive signal, however, could be detected on day seven after challenge in the BALF collected from animals vaccinated with NG34+CAF®01 (Figure 3.5F).

Subunit vaccines did not achieve seroprotective titres before challenge in comparison to STIV

HI and NT antibody titres were evaluated in the sera and BALF collected from vaccinated and non-vaccinated animal groups 7 days after the challenge with pdm09 H1N1. NT titres were either negative or low in non-vaccinated challenged animal group. All other groups vaccinated with NG34+CAF®01, NG34+CDA/αGCM, STIV or NG34+FA showed a positive reaction in the NT assay albeit with lower intensity and variations within and among the groups. However, STIV vaccinated animals showed higher NT titres in comparison with the rest of experimental groups (Table 3.3).

Table 3.3. HI titres in sera and neutralization titres in BALF from individual animals euthanized at 7 dpi (3 animals in each group except for the NG34 + FA group, with 2 pigs).

Experimental Group	HI Antibody Titre in Sera *	Neutralization Antibody Titre in BALF *
PBS-NV/C	80	20
	80	40
	80	30
NG34+CAF®01	320	0
	80	30
	160	120
NG34+CDA/αGCM	160	80
	160	40
	80	20
STIV	160	120
	640	30
	2560	100
NG34+FA	160	30
	320	30

* Titres expressed against challenge strain A/Catalonia/63/2009 pdmH1N1.

HI antibody titres, on the other hand, were increased a bit in the non-vaccinated challenged control animal up to 80 due to the effect of pdm09 H1N1 infection. HI titres in the rest of experimental groups were in general increased (2–3 fold) but outstandingly in STIV vaccinated animals (14-fold in proportion comparing with NV/C group) (Table 3.3).

Discussion

Adjuvant formulations are necessary components of modern vaccines based on subunit proteins/peptides, which are often poorly immunogenic without additional immune stimulants [121]. On the other hand, different antigen structures may be affected by adjuvant formulations such as emulsions. For instance, virus-like particle antigens may interact with adjuvant formulations in very different ways compared with recombinant subunit proteins or immunogenic peptides [178]. The use of adjuvants can also result in a skewing of the resulting humoral or cellular immune response [179,180]. This can in turn improve or reduce vaccine efficacy or even promote immune pathological reactions (e.g., antibody dependent enhancement (ADE), vaccine-associated enhanced respiratory disease (VAERD) [122,181]).

In this study, we assessed the immune responses to pdm09 H1N1 IV infection in pigs vaccinated with HA1 peptide NG34 adjuvanted with CAF®01 or CDA/αGCM. The NG34-specific IgG response was significantly elevated in the sera collected from pigs vaccinated with NG34+CAF®01 and was observed before and after challenge with pdm09 H1N1 IAV. Both the IgG1 and IgG2 isotypes were produced after vaccination with NG34+CAF®01 being IgG2 isotypes more dominant than IgG1, in contrast to infection with live virus that generated a more balanced and broader immune response. Strong IgG2 response was observed also in a previous study in mice immunized with NG34+CAF®01 [97] and it is in line with other recently published studies using combination of different antigens with CAF®01 [131,135].

Previously, it has been suggested that IgG2 response is vital in protection against IV, particularly in the absence or low amount of virus neutralizing antibodies [182,183]. Pigs vaccinated with the NG34+CAF®01 combination also generated relatively good HI titres although variations within the animal group was observed. Similarly, the NG34+CAF®01 vaccinated group also showed NT titres in BALF, albeit with variations and at low levels. More importantly, NG34+CAF®01 vaccinated group showed relatively lesser pulmonary lesion scores and reduced virus load in the BALF as well as lower virus shedding after pdm09 H1N1 infection. These results are in line with the ones obtained in ferrets with TIV combined with CAF®01, where protection against heterologous IAV was observed in an HIA-independent manner [171]. NG34-specific IgG titres in pigs vaccinated with NG34+CDA/αGCM were relatively low. Moreover, no NG34-specific IgG1/IgG2 isotype response was observed in pigs vaccinated with this latter combination, except one animal that showed relatively higher IgG titres in sera collected both at pre- and post-challenge time points in this group. However, this group was the only one showing a statistically significant reduction of viral load in BALF on day three pi. This can be explained, at least in part, by the fact that NG34+CDA/αGCM and STIV were the only groups in which it was observed a significant increment in HA1-specific antibodies in BALF, as well as H1N1 and HA1 specific IFNγ-producing cells. In terms of lung pathology, this group immunized with NG34+CDA/αGCM combination had a very variable pathological score, even higher in some individuals than the non-vaccinated challenged group. This was also

true, to a lesser extent, for the animals in the STIV and NG34+FA groups. Interestingly, similar outcomes, including an increased viral shedding were reported in a pig experiment with animals immunized intranasally with αGCM before a challenge with pandemic H1N1 A/California/04/2009 [184]. On the other hand, CDA-adjuvanted vaccines against IV have provided efficient protection [185]. The so-called VAERD effect is defined as an undesirable side effect described in pigs with some inactivated-based Influenza vaccines, characterized by an exacerbation of the severity of the IV induced disease, including long lasting fever, clinical signs and an increase of lung consolidated areas [186]. Despite the fact that we cannot relate these extended pulmonary lesions to a VAERD effect due to the low number of animals used in this study, we considered that this is an issue to further study in regards the αGCM/antigen combination.

In response to in vitro stimulation of PBMC with inactivated pdm09 H1N1 influenza virus, we detected a peak of IFNγ-producing cells in all vaccinated pigs, including with the STIV vaccine, 7 days after challenge with the pdm09 H1N1 virus. The lack of IFNγ response observed in pigs vaccinated with the non-adjuvanted TIV vaccine (even following the booster vaccination) reflects the incapacity or at least the low efficiency of the non-adjuvanted vaccines to elicit an influenza-specific lymphocyte T response in these pigs as it was reported previously in ferrets [136]. Such an anamnestic response in the number of influenza-specific IFNγ-producing cells in the blood has similarly been detected only at day 7 after the challenge of pigs with A/Sw/Indiana/1726/88 H1N1 swine influenza virus [187]. It has been previously shown that

some adjuvants have the ability to strongly enhance antigen cross-presentation (including that of peptide or protein antigen) [188–190]. CAF®01 adjuvant contained in the adjuvanted NG34 vaccine induce local inflammation and recruitment of various innate immune cells as has been reported for other adjuvant ASO3 [123], although their mechanism of action is different in terms of depot formation [138]. This depot formation produced in the tissue by CAF®01 induces a strong cell response involving CD4+ T cells [191] and may similarly enhance antigen cross-presentation with the subsequent CD8+ cytotoxic T cell proliferation. Only NG34 combined with CAF®01 vaccine elicited systemic humoral responses as well as an enhanced cell-mediated immune response 7 days after pdm09 H1N1 challenge. However, pigs vaccinated with this combination, although having reduced viral load and cleared the virus earlier, were unable to significantly limit nasal virus secretion, as viral RNA continued to be detected in the nasal cavity. In this respect, nasal shedding progressed as expected in a similar way as it occurs in untreated animals [192].

It has previously been shown that pigs with an HI antibody titre equal to or above 20 were generally protected from a subsequent influenza challenge [193]. Even though all vaccinated animal groups (CAF®01, CDA/αGCM, FA, STIV) exhibited HI titres above 40, they were not significantly protected. It is still unclear how the systemic responses generated after vaccination correlate with local mucosal responses in the respiratory tract that may also contribute to reduction in virus shedding [194]. Interestingly, a similar study conducted in ferrets challenged six weeks after the initial vaccination, generated results

consistent with our own findings with NG34+CAF®01 vaccine. This study showed that pdm09 H1N1 vaccines reduced (adjuvanted split vaccine) or had no effect (non-adjuvanted whole vaccine) on the viral shedding from the upper respiratory tract, although the adjuvanted split vaccine did prevent viral replication in the lower respiratory tract of ferrets [195,196]. This suggest that local immunity may play a role for viral shedding but less for pulmonary disease. This could, e.g., be obtained by priming parenterally to obtain the systemic immunity important for prevention of pulmonary disease and boost intranasally to obtain local immunity in the nasal cavity and thus avoid viral shedding. This prime-pull strategy has previously been described for CAF®01 in mice [197] and pigs [198].

Immunization with NG34+CDA/αGCM under the current experimental conditions, on the contrary, appeared less effective than immunization with NG34+CAF®01 both in inducing adequate immune response and limiting pathological outcome after pdm09 H1N1 challenge. Specific NG34 anti-IgG were only observed in one animal of the group immunized with NG34+CDA/αGCM before and after the IAV challenge. Similarly, increased number of viral particles in nasal swabs during the experiment and in BALF collected on day seven after challenge as well as extended lung consolidated lesions on day three after the infection with pdm09 H1N1 IV were observed in this group. These findings contrast to the ones from a study published by Khatri et al. [199], where protective effect of αGalCer adjuvant, a component also included in the adjuvant used in our study, against Swine Influenza Virus (SwIV) infection in pigs has been demonstrated. In this study,

however, the authors used a UV-inactivated SwIV together with αGalCer as vaccine. Thus, the CD1d agonist αGCM in combination with CDA might be a suboptimal adjuvant for a short peptide-based vaccine. Moreover, the route of application (intranasal), age and type of piglets and particularly the concentration of αGalCer used in this study was also different to the one presented here. The authors could document that protection against SwIV infection in pigs vaccinated with UV-inactivated SwIV + αGalCer correlated with the αGalCer concentration used. Likewise, another study from Artiaga et al. [200], reported that αGalCer protects pigs from IV infection when administered as vaccine adjuvant and attributed the observed protection to enhanced NKT-cell concentrations resulted after administration of vaccine containing αGalCer. A more recent study report by Gu et al. [184], in contrast, suggested that increased NKT-cells does not alter disease outcome in pigs prophylactically treated with αGalCer. In all these reports, unlike our study, αGalCer was either used alone or as an adjuvant with antigen combination. At this stage, we cannot rule out that the combination of CDA/αGCM used in our study with NG34 might have an antagonistic effect on activation of immune cells, as also suggested in a study published by Matos et al. [201] demonstrating that protection against *Trypanosoma cruzi* infection in mice is more efficient when only CDA rather than αGCM is used as adjuvant together with Tc52 antigen. In all cases, this hypothesis needs further research to be ascertained. For this reason, we consider that optimization of dose in the combination of CDA and αGCM adjuvants would be needed in order to better determine its efficacy and immunogenicity.

In summary, this study helped to report the outcomes in pathology, cell-mediated and humoral immune responses resulting from the vaccination with the novel vaccine formulations and the subsequent infection. Unfortunately, NG34-combinations formulated for this study did not afford the immune correlates of protection achieved by the STIV, nowadays accepted for the licensed vaccines. However, we admit that the number of animals per group used in our study was rather small to make statistically relevant conclusions. A higher number of parameters like route of application, age of animals, concentration of adjuvants, antigen choice, different challenge strains and in particular, sufficient number of animals should be considered in future experiments. These issues will help in the future to obtain conclusive and statistically significant results to dissect the relevant parameters for the induction of protective immune responses against IV infection in pigs.

Conclusions

Pigs vaccinated with NG34 adjuvanted with CAF®01 or CDA/αGCM reacted differently upon IAV infection regarding pathological outcome, viral shedding, and cell-mediated and humoral responses. Thus, pigs immunized with NG34 in combination with CAF®01 seroconverted against the antigen, had numerically lower lung lesion score, decreased viral shedding and increased of IFNγ producing cells. On the contrary, pigs immunized with NG34+ CDA/αGCM elicited a weak immune response (both humoral and cell-mediated) without correlation with

protection. However, further studies scaling-up the number of animals would be needed to confirm the effectivity and immunogenicity of these combinations of adjuvants and antigens against IAV infection.

Chapter IV. Study II.A. Immune responses following neonatal vaccination with CAF®01 or CDA adjuvanted conserved fragment F4 against virulent *Glaesserella parasuis* and the effects observed on its natural colonization.

Chapter 4. Study II.A. Immune responses following neonatal vaccination with CAF®01 or CDA adjuvanted conserved fragment F4 against virulent *Glaesserella parasuis* and the effects observed on its natural colonization.

Abstract

Glaesserella parasuis is a Gram-negative bacterium that colonizes the upper airways of swine and is capable to cause a systemic infection called Glässer's disease. Current treatments against *G. parasuis* infection are centered in antimicrobials or inactivated vaccines, which have limited cross-protection against different serovars. For this reason, there is an interest in novel subunit vaccines that confer an effective immunity against different virulent strains. In this study, we aimed to test the immunogenicity of the F4 protein (a conserved fragment found in the trimeric autotransporters from virulent *G. parasuis* strains previously characterized as immunogenic) along with the immunostimulatory adjuvants CAF®01 and CDA in neonate piglets. Twenty-three piglets were distributed into 4 groups, including two adjuvanted F4 groups, a vaccinated with an inactivated product one and the non-vaccinated control. The animals were twice immunized from their second week of life in an interval of 21 days. Blood samples were taken during the study to assess the humoral and cell-mediated immune responses. F4+CDA vaccinated piglets triggered a strong specific anti-F4 response biased to IgG1 subtype in comparison to the rest of the groups. Moreover, vaccinated animals with either subunit vaccines or bacterin displayed

an increase of central and effector memory cells upon *in vitro* re-stimulation with F4, with a Th1-type response. Specific anti-F4 maternal antibodies transferred via colostrum were detected in the neonate piglets at the beginning of the study. This fact, together with the detection of nasal colonization with serovar 4 of *G. parasuis* during the experiment, affected the immune profiles of each animal, especially the animals immunized with F4 + CAF®01. However, most of these latter animals were able to control and clear out the colonization in the absence of detectable antibodies or systemic specific F4 response, indicating a primed mucosal cell-response. According to the results, the exerted immunogenicity of the vaccine formulations depended on the antigen and adjuvant combination and F4 may represent a good candidate to consider against Glässer 's disease.

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 [53]. *G. parasuis* comprises several serovars that can be pathogenic to pigs, especially in the post-weaning period. At that age, around 4-8 weeks of life, susceptible piglets may develop invasive *G. parasuis* disease, also known as Glässer's disease, which is characterized by fibrinous polyserositis, arthritis and meningitis. Glässer's disease causes serious economic and animal welfare problems to the swine industry [7]. *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 this disease [85].

Neonate vaccination is an effective tool for the protection against infectious diseases that are common in the early stages of life [202]. However, newborn animals have an immature immune system, and protective immunity in these early stages of life can be difficult to be achieved due to the skew of the immune system towards Th2, Treg responses and limited Th1 and antibody responses [31,32]. 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 [203,204] as well as the microbiota that colonize their mucosae [205,206]. Current vaccines against Glässer's disease consist of autogenous or commercial bacterins, which confer protection against homologous or a narrow heterologous range of serovars [83]. 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, caught our attention [100,101]. Comparison of the amino acid sequence among the VtaAs from strains with different degree 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* [107]. Recent work, also 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 [108].

Aiming to extend these studies, here we explored the vaccine potential of the F4 protein fragment in neonate pigs, in this occasion testing the efficacy of two novel adjuvants: the Cationic Formulated 01 (CAF®01) and the bis-(3,5)-cyclic dimeric adenosine monophosphate (CDA), specifically designed to stimulate different immune pathways. On one hand, CAF®01 is a liposome-based mixture of the ammonium surfactant N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) and the synthetic glycolipid analogue to the *Mycobacterium tuberculosis* cord factor α,α'-trehalose 6,6'-dibehenate (TDB), and it was chosen due to its ability to promote Th1/Th17-like responses [131,135]. On the other hand, CDA, a monocyclic dinucleotide naturally secreted by *Listeria monocytogenes*, was selected due to its ability to efficiently activate STING, the "Stimulator of Interferon Genes", triggering balanced Th1/Th2/Th17-like responses [146,185].

In this vaccination study we assessed the humoral and cell-mediated immune responses in neonate piglets following the immunization with F4 formulations. For this purpose, specific antibodies and T-cell responses were measured as well as the effect of a secondary colonization by a heterologous strain of *G. parasuis* in these parameters.

Material and methods

Ethics regulation

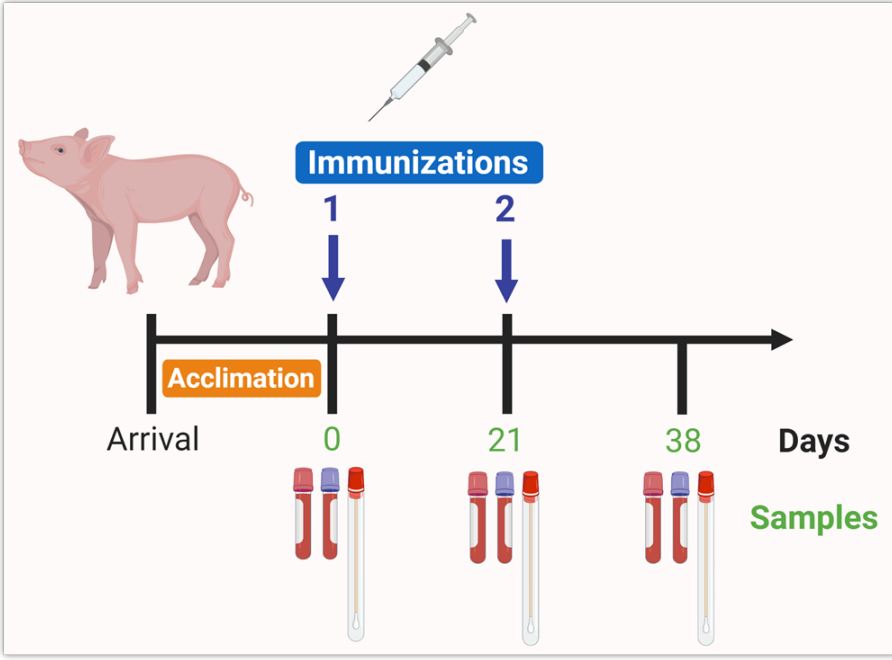
Animal experiments were conducted in AM Animalia (La Vall de Bianya, Girona, Spain) according to the ARRIVE and the Declaration of Helsinki guidelines, approved by the AM Animalia Ethics Committee for Animal Experimentation with number CEEA 20/20-P1 and CEEA 20/20-P2 in compliance with the EU directive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

Experimental vaccines preparation

F4 immunogen 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 37ºC 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 the F4 protein. 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. 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 protein was stored at -20ºC 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 as indicated in Figure 4.1, injecting 100 μg of immunogen in a total volume 500 μL per dose intramuscularly in the neck of the piglets. Two additional piglets were immunized with the Porcilis® Glässer vaccine, composed of inactivated serovar 5 *G. parasuis* adjuvanted with dl-α-tocopheryl acetate, as controls for the *in vivo* assay, following the recommendations of the manufacturer (MSD Animal Health, Salamanca, Spain).



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.

Figure 4.1. Schematic representation of in vivo experimental procedure and group distribution of the experimental vaccines. Piglets from 3-4 days of age were transported to the experimental facilities and left for acclimation for one week. 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 first immunization, prior to boosting) and 38 (17 days after boosting. Figure created with BioRender.com.

Experimental design

The study design is displayed in Figure 4.1. Twenty-three Landrace x Duroc piglets of about five days-of-age were included in the study. Piglets were selected from 8 sows between parities 3-5 with negative (5), doubtful (2) and positive (1) antibody values against *G. parasuis*

according to the commercial Ingezim–Haemophilus ELISA (Ingenasa, Madrid, Spain) and the lowest measured by an *in-house* ELISA against F4 protein (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, distributed into four pens, one group in each pen including piglets from all the sows in every group. Animals were fed *ad libitum* during all the experiment and treated with 5mg/kg of ceftiofur (Naxcel porcino, Zoetis S.L.U., Spain) during the first two weeks of the study to prevent undesirable bacterial diseases. After seven days of acclimation, animals were intramuscularly vaccinated in the neck on day 12 of life (Day zero of the study; D0) and twenty-one days later, piglets were boosted with a second dose of each vaccine (D21) alternating the vaccination side in each injection. 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 x *g*) of clotted-blood tubes were aliquoted and stored at -80ºC. Nasal swabs were resuspended in 500 µL of PBS and stored at -80ºC 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. Virulent and non-virulent *G. parasuis* strains were differentiated by PCR using two primer sets that differentially amplify their *vtaA* leader sequences (LS-PCR)

[104]. Four μL of the DNA extracted from the nasal swabs were used as 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 using the DNA purified from the nasal swabs using a serotype-specific PCR [207], 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 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. 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 to 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 from all animals at 21 and 38 days postvaccination (DPV) and additional PBMCs from D0 control naïve pigs (prior to PBS or Porcilis® Glässer injection) were additionally used to determine the basal detection of IFN-γ secretory cells at the starting point. 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% foetal 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 in Chapter III, 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 later with 25 ng of HRP-streptavidin (Invitrogen Life technologies. Madrid, Spain) both per well; followed by a development with 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 with F4 as described above, and ten micrograms per mL of concanavalin A (Sigma-Aldrich, Madrid, Spain) were used as a positive control. Supernatants were collected and stored at -80ºC for cytokine analysis. PBMCs were harvested and washed with FACS buffer (0.5% FBS-PBS). After washes, cells were surface stained for 45

minutes at room temperature with a mixture of labelled antibodies (Table 4.1) at the indicated dilution 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 1.

Table 4.1. List of antibodies used for immunophenotyping of stimulated PBMCs using surface marker labelling by flow cytometry.

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

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 valid statistical analysis, data were screened for unlikely or 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 38 DPV (yes vs no), sow origin (n=8 sows), sow parity (3, 4 and 5), total anti-*G. parasuis* antibody ratio (doubtful, positive and negative 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 38 DPV/ anti-F4 IgG levels in sow, ELISPOT Nagasaki 21 DPV, 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 48h of stimulation with the F4 protein. 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. 2009 [208].

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). Signification 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 2007 [209] and Richardson 2011 [210]. The confidence interval was calculated according to Altman et al. 2000 [211]. Graphs were plotted using Prism v9 (GraphPad Software, San Diego, CA, USA).

Results

Piglets became positive to a virulent-related serovar 4 of G. parasuis during the vaccination study

A non-desired nasal colonization by virulent serovar 4 *G. parasuis* was confirmed by PCR at D0 (before immunization started) in four unvaccinated control animals (Figure 4.2). Further PCR analysis 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 in PBS, 7/7 in F4+CAF®01, 5/7 in F4+CDA and 1/2 in Porcilis® Glässer). Later, the colonization by serovar 4 virulent *G. parasuis* was significatively reduced in the F4+CAF®01 immunized pigs

(p=0.02), with two animals being PCR-positive at D38 out of the seven detected at D21. The reduction of the virulent / serovar 4 *G. parasuis* was not that evident in the rest of the groups (Figure 4.2). Individual outcomes are available in the appendix (Supplementary table 5).

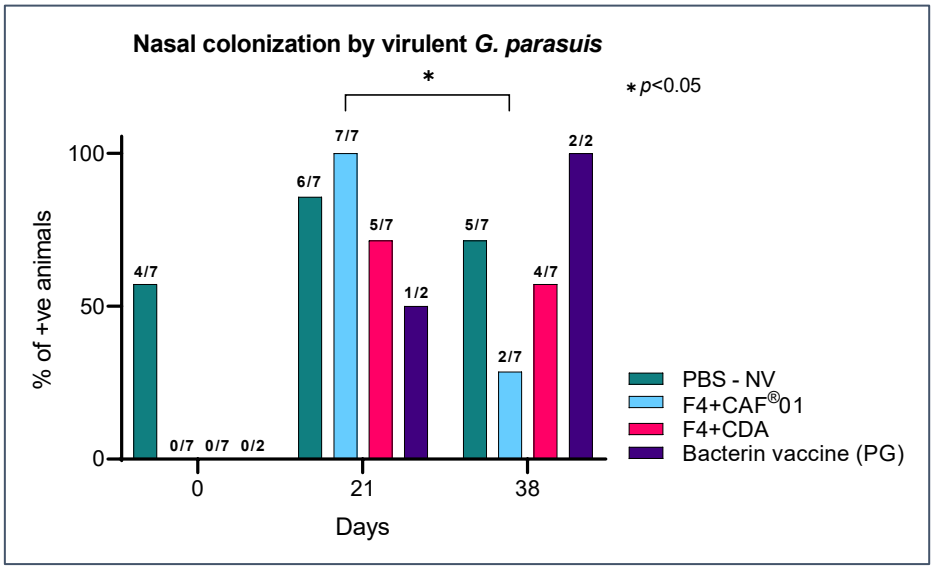


Figure 4.2. Nasal colonization of the piglets by virulent G. parasuis during the vaccination experiment measured by LS-PCR from nasal swabs collected at 0, 21 and 38 DPV. Bars represent the percentage of positive animals to virulent strains within the group in the different timepoints. Above each bar 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.

F4+CDA vaccinated pigs elicited significantly higher anti-F4 humoral response compared to those vaccinated with 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 boosting (D38). As shown in Figure 4.3A, animals vaccinated with F4+CDA showed significantly higher antibody levels on D38 than the PBS-immunized control pigs ($p<0.01$). Similar results were found for the anti-F4 IgG1 immunoglobulin isotype (Figure 4.3B), but not for the anti-F4 specific IgG2 (Figure 4.3C) and IgM (Figure 4.3D). At D0, total IgG and IgG2 antibody levels against F4 (Figure 4.3A) were more variable in the new-born piglets from the unvaccinated group. Surprisingly, by D38, the level of anti-F4 specific IgG, IgG2 and IgM (Figure 4.3A, 4.3C and 4.3D, respectively), but not for the IgG1 isotype (Figure 4.3B), slightly increased in the unvaccinated control group, indicating a response probably due to the undesirable infection by the *G. parasuis* virulent strain mentioned above. The individual kinetic profiles of anti-F4 specific antibodies are available in the appendix (Supplementary figure 2).

Variable and increased values of total anti – *G. parasuis* were found at 0DPV in all the groups. Only the two animals immunized with the bacterin vaccine Porcilis® Glässer showed an rise of total anti – *G. parasuis* antibodies on 38DPV (Supplementary figure 3).

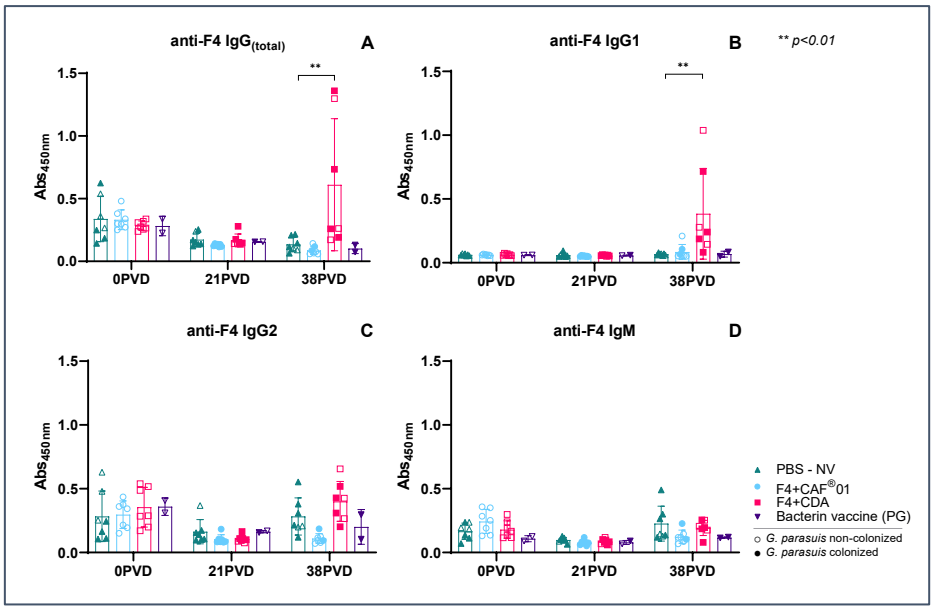


Figure 4.3. F4-specific antibodies detected in sera from pigs immunized with either F4+CDA, F4+CAF®01, PBS or Porcilis® Glässer along the experimental procedure from days 0. 21 and 38 DPV. Anti-F4 specific antibody kinetics: Total IgG (A), IgG1 (B) IgG2 (C) and IgM (D). DO obtained for each pig are plotted with solid symbols, averages and standard are also plotted. Statistical significance (*) and p values are indicated where it corresponds.

Different responses between groups were detected in F4-specific antibodies and IFN-γ secreting cells

F4 specific T-cell responses were assessed in the different immunization groups using an IFN-γ ELISPOT (Figure 4.4). Specific secretion of IFN-γ by F4-stimulated PBMCs was already detected by ELISPOT in some piglets on D0, probably indicating maternal transfer of F4-specific T-cells to their offspring. The number of F4-IFN-γ specific spots notably increased in all groups by D21, independently of the treatment received coinciding with the peak of virulent *G. parasuis* detection in the nasal cavity. After boosting, levels of IFN-γ secretion decreased generally, but

was slightly retained in F4+CDA vaccinated animals (Figure 4.4). A general trend was detected with colonized-negative piglets having lower IFN-γ response, and in fact colonization status at D38 was significantly associated with the IFN-γ results in the ELISPOT with bacterin at D21 (not shown); i.e., the piglets with virulent *G. parasuis* in the nasal cavity at D38 had higher IFN-γ response against the whole bacterium (22.00 [-17.95 - 61.95], p=0.0021).

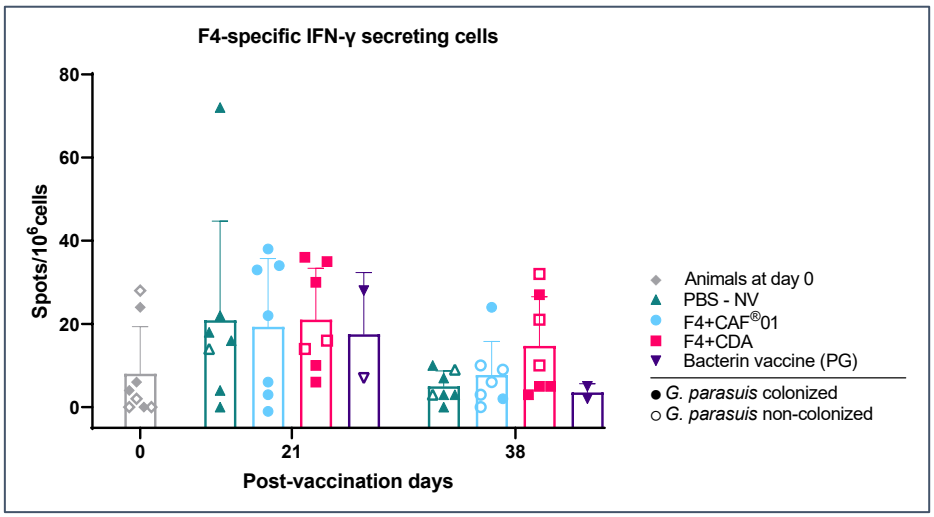


Figure 4.4. F4-specific IFN-γ secreting cells measured by ELISPOT. PBMCs (5x10⁵) collected at different times during the study were plated and stimulated for 48 hours with 2 μg/ml of F4. Individual piglets colonized at each timepoint by virulent G. parasuis are represented with filled dots, whereas non-colonized animals are represented with empty dots. Averages and standard deviations found in each group are also plotted. On day 0, as piglets were naïve and not still treated, all tested animals are represented in the same bar.

A closer look to the net individual responses for anti-F4 IgG and IFN-γ secretion revealed that even though strong values of specific antibodies

were found in the pigs vaccinated with F4+CDA formulation, this fact was not reflected in more IFN- γ secreting cells, since only three animals of this group showed this increase. On the other side, the net increase of F4 specific antibodies in some piglets of the unvaccinated group and specifically for the pigs numbers 1.2, 1.3, 1.5 and 1.7 (Figure 4.5), indicates that both immune parameters (F4-specific antibodies and IFN-γ secreting cells) are potential indicators of colonization by virulent *G. parasuis*, rather than response to vaccination.

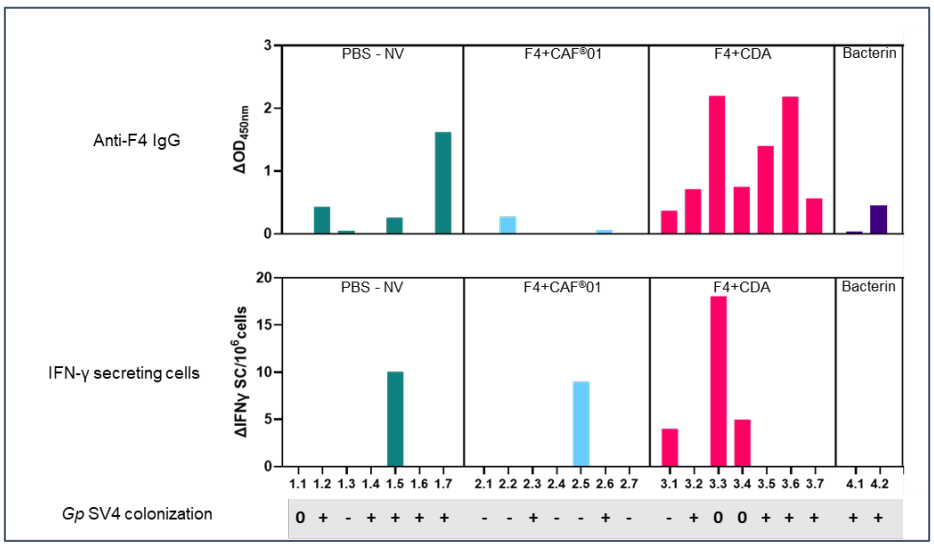


*Figure 4.5. Increase in F4-specific immune responses from D21 to D38 induced by the different immunization regimes. F4-specific antibody (above) and IFN-γ secreting T-cells (below) found in individual pigs immunized with either F4+CAF®01, F4+CDA, PBS or Porcilis® Glässer. Δanti-F4 IgG result from subtracting 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. At the bottom of the figure the nasal colonization by G. parasuis is indicated by symbols: "-"D38 non-colonized pigs; "+" D38 colonized pigs, and "**0**" pigs that were not colonized at any time along the experiment.*

Vaccinated piglets showed a trend of higher activation of different T-cell subsets and the induction of anti-inflammatory cytokines in response to in vitro stimulation with the F4 protein.

Although not statistically significant between groups, some positive trends were observed in the F4-specific T-cell subsets found by day 38 in uncolonized animals. Higher proportions of F4-specific double positive CD4+CD8+ (memory T-cells), CD3+CD4+CD8+CD27- and CD3+CD4+CD8+CD27+ (effector and central memory T cells, respectively) were found in F4 vaccinated piglets, whatever the adjuvant used, in comparison to the unvaccinated group (Figure 4.6). Conversely, the colonized animals from non-vaccinated group (PBS) showed high and variable proportions of memory T cells and Th helper cells after F4-*in vitro* stimulation than non-colonized pigs. Colonized piglets vaccinated with F4+CAF®01 combination showed lower proportions of memory T-cell subsets, whereas the F4+CDA immunized animals displayed variable proportions in the mentioned lymphocyte populations (Figure 4.6).

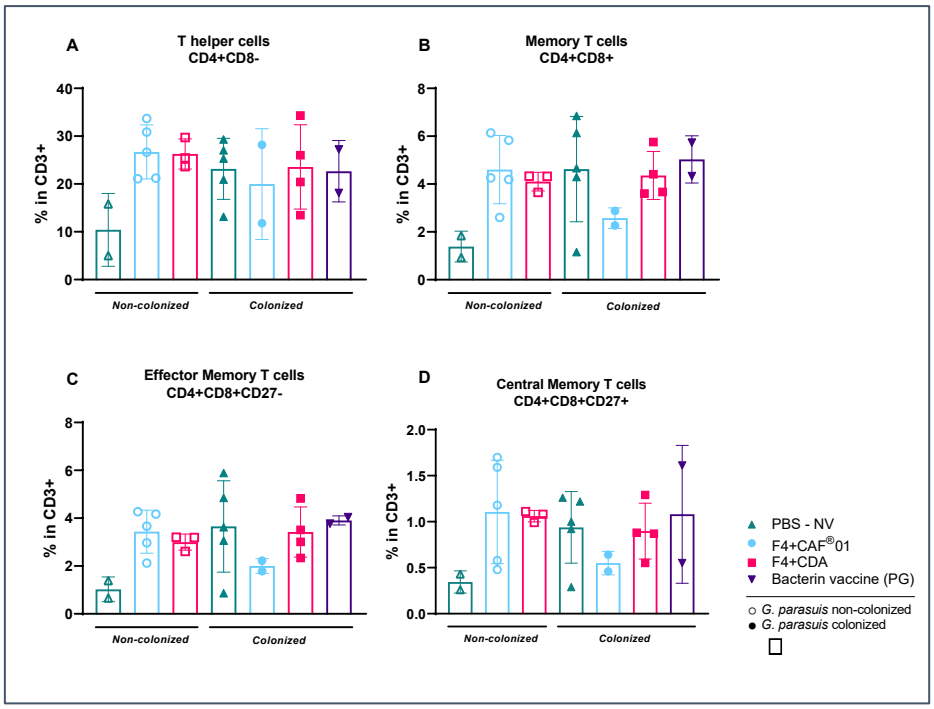


Figure 4.6. Proportion of T cell populations found on in vitro F4-stimulated PBMCs collected on day 38 and analysed by flow cytometry. Results are expressed after gating of twenty thousand events acquired on total PBMCs. Individual piglets colonized at this timepoint by virulent G. parasuis are represented with filled dots, whereas non-colonized animals are expressed with empty dots. Averages and standard deviations found in each group are also plotted.

No significant differences were detected in the cytokine responses among the different groups, probably due to the high intra-group variability. However, *in vitro* stimulation with the F4 protein displayed some minor tendencies in cytokine levels (Figure 4.7). Cytokines associated with a Th1 response, such as IL-1β, IL-12p40 and IFN-γ, became increased upon the stimulation with F4 protein in F4+CDA vaccinated animals, while IL-6 and TGF-β appeared slightly increased in non-colonized animals vaccinated with F4+CAF®01 suggesting the

stimulation of a Th17-type response (Figure 4.7). Conversely, the colonization with serovar 4 of *G. parasuis* on D38 affected positively the secretion of IFN-α upon the stimulation with inactivated Nagasaki (p=0.06), whereas the expression of TGF-β seemed to be downregulated in the colonized animals (p=0.05).

Maternal immunity and colonization affected the immunological outcome observed in all groups of pigs

The multivariable statistical model performed to evaluate possible relations among variables showed a significant association between the amount of IFN-γ secreting cells of the piglets and their sow origin. According to the results, the sow and their specific anti-F4 IgGs affected significatively the IFN-γ secreting PBMCs of the offspring before the boosting (21 DPV) with p values of 1.36e-08 and 0.02556, respectively. Moreover, the final model showed a significant association between the secreted IFN-γ on day 21DPV, and the colonization by virulent *G. parasuis* detected 17 days later with a p value of 0.0021.

The statistical model additionally showed that 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, the statistical model also revealed that secreted TGF-β by PBMCs is positively affected by the anti-F4 specific antibodies found in the sow of origin (p<0.05).

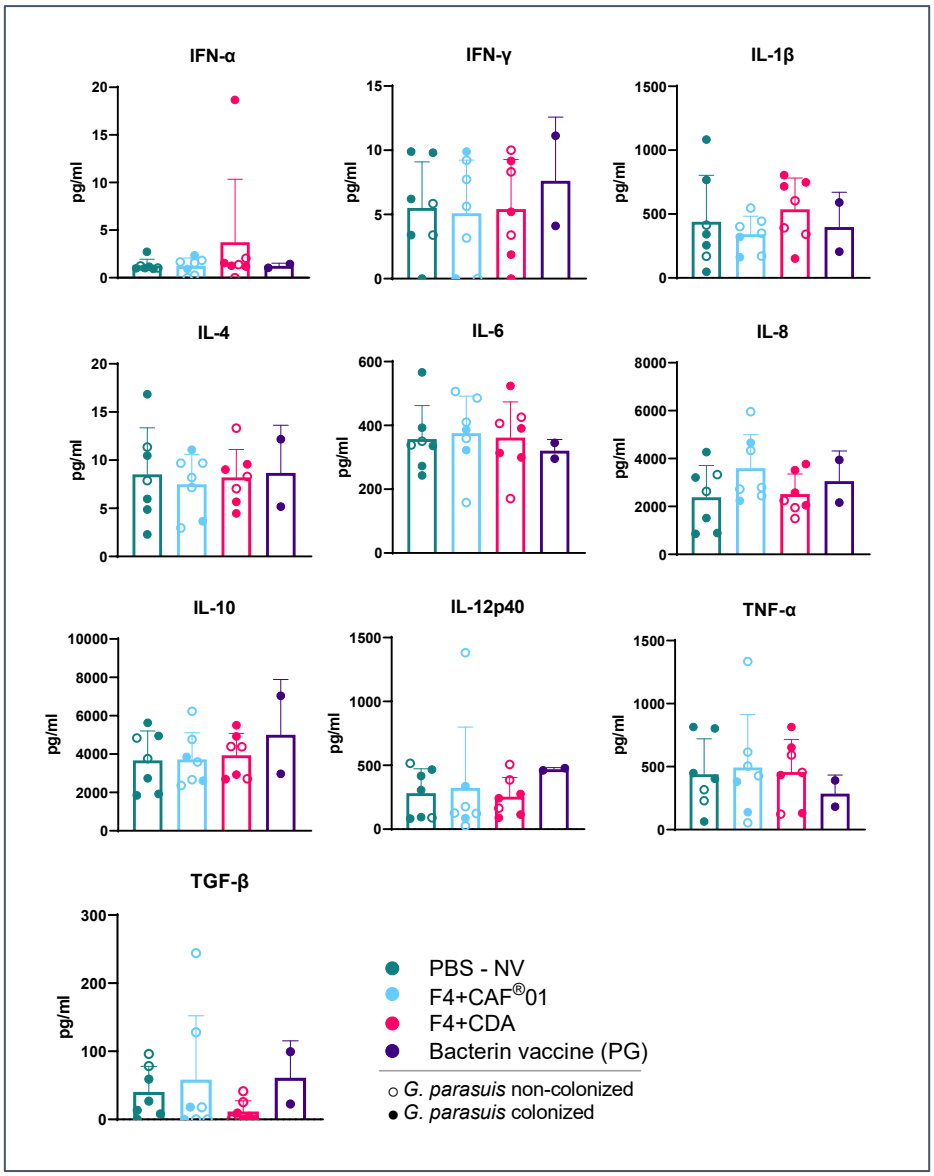


Figure 4.7. Cytokine profiles obtained by ProcartaPlex™ immunoassay and TGF-β ELISA from the supernatants of F4- stimulated PBMCs on day 38 from all pigs. Individual pigs colonized on this time point by virulent G. parasuis are represented with filled dots, while non-colonized animals are expressed with empty dots. Averages and standard deviations found in each group are also plotted.

Discussion

In this study we describe the immune responses in young piglets vaccinated with a conserved immunogen of virulent *G. parasuis*, the F4 protein fragment, combined with the adjuvants CAF®01 or CDA. However, the immune responses elicited by the animals were highly variable within groups, and we identified parameters of maternal influence (levels of anti-F4 antibodies and sow parity) and the colonization status of the piglets with serovar 4 virulent *G. parasuis* as the sources of this variation. The animals immunized with the CDA combination elicited an efficient humoral response that did not correlate to protection against a natural colonization. CAF®01 vaccinated animals on the contrary, were able to clear the colonizing bacteria from their noses without inducing detectable specific antibodies or T cell responses, indicating a primed mucosal response.

Certainly, the results observed in the unvaccinated group, with F4-specific antibodies during the experiment and IFN-γ secreting cells in early stages, suggested an infection by *G. parasuis*, which was subsequently detected as colonization by serovar 4 virulent *G. parasuis*. Thus, in the unvaccinated group we observed piglets with maternal immunity that remained protected from the colonization during the study, animals where the colonization elicited an immune response, and animals non-colonized that did not generate immune responses against *G. parasuis*. Moreover, we were able to identify one animal from the control group that cleared the colonization detected at 21DPV and showed the lowest proportion of F4 memory T cells and high quantities of anti-inflammatory cytokines. This detailed examination of the

unvaccinated animals confirms the lack of a real negative control in this study, but this fact may correspond to a similar scenario to that under farm conditions.

Despite the high individual variability of the piglets, the experimental vaccine formulations were able to elicit different immune responses in the piglets, an important fact considering the young age of the animals. CDA formulation significantly improved the induction of specific IgGs (mainly from the subclass 1) against the F4 protein by D38 (17 days after boosting), when compared with the rest of the immunization groups. Similar outcomes in humoral response were previously reported with STING agonist (CDA or cyclic di-GMP (CDG)) adjuvanted antigens from diverse pathogens injected in mice [212–214]. The delivery system of the vaccine formulation might affect also the development of a proper mucosal response, as reported in pigs vaccinated with a *Mycoplasma hyopneumoniae* bacterin adjuvanted with liposome-encapsulated CDA [215]. In our study, only one animal from the F4+CDA vaccinated group was able to clear the colonizing serovar 4 *G. parasuis* from the nasal mucosa. This fact indicates that F4-specific systemic antibodies may not correlate with a putative protection against natural colonization by virulent *G. parasuis* (serovar 4), in agreement to the results observed with antigens from similar bacteria [216–218]. Nevertheless, the vaccination with bacterin formulation Porcilis® Glässer adjuvanted with α-tocopheryl acetate, did not avoid the natural colonization of serovar 4 of *G. parasuis* even eliciting antibodies against the whole bacteria.

The piglets vaccinated with F4+CAF®01, on the contrary, did not elicit a significant humoral response against the immunogen. Only two

animals showed a low F4 antibody signal that we might attribute to the colonization in these two animals. The lack of antibody induction 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. [219]. 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 of cyclic dinucleotides to an autonomous activation of B cells through the STING signaling pathway [220].

Humoral response and, in particular, the generation of opsonizing antibodies, is considered crucial for the protection against Glässer's disease [221]. In this respect, maternally derived immunity plays an important role in the offspring protection by the transference through the colostrum of high amounts of antibodies that can opsonize bacterial pathogens and protect in the first stages of life [222]. However, maternal immunity may also affect the vaccine response in neonate piglets, obstructing the proper activation of B cells at least in their first weeks of life. CAF®01 and CDA adjuvants proved a complete efficacy and immunogenicity in previous neonatal studies performed in mice [139,223]. 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. In the case of the CAF®01 formulation, besides the effect in the humoral response of the pre-existing maternal antibodies, we 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 [224]. Hence, the authors related efficient Th1/Th17-type responses to higher adsorption rates of the antigen into the vaccine formulation, in inverse correlation with antibody titres.

In our study, the colonization with serovar 4 of *G. parasuis* offered the opportunity to evaluate the effectivity of the different vaccine combinations under a natural exposure to the pathogen. In these regards, only CAF®01 combination was significatively able to reduce the nasal colonization by *G. parasuis*, in agreement with the Study I with Influenza virus in lung (Chapter III) and previously reported with both viral and bacterial pathogens [198,225]. This clearance may indicate a primed mucosal response due to the adjuvant effect characterized by Th1/Th17 type responses. was observed, the elicited mucosal response can be useful against the colonization of pathogenic respiratory bacteria. Our results also show that the extent of the maternal influence in vaccination response is not only restricted to the humoral immunity. Lymphocyte subsets evaluated by flow cytometry showed highly variable results. In fact, excluding the animals colonized by serovar 4 of *G. parasuis* on day 38DPV, we observed different levels of T helper and memory T cells. These results might indicate that the Th responses of the adjuvanted combinations with F4 were not affected by the presence of maternal antibodies. It would be tempting to suggest that an increase of the number of piglets in the groups would have given significance to these results. Furthermore, we could speculate that colonization can

modulate the cell-mediated immunity as it can be observed within the unvaccinated group in the afore mentioned T cell subsets. Importantly, the multivariable model indicated correlation between sow parity and amount of T helper and memory T cells in the piglets, as recently reported after a cell-subset assessment in sows of diverse parities [226]. Like the lymphocyte subsets, the cytokine profile displayed by stimulated PBMCs was variable, with a trending towards a Th1/Th2 bias in the piglets vaccinated with CDA. Again, the variability within vaccinated groups impeded to obtain conclusive results. However, the multivariable 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 with 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 protein were upregulated in the offspring of F4-vaccinated sows [108]. This trait, together with a slight increase of Th17-like response cytokines, might indicate an intrinsic immunogenicity of the vaccine protein that requires further attention. Although it is not yet described, we cannot rule out that F4-protein may contain Treg or Th17 epitopes as it was recently described for the passenger domain of the complete *vtaA* protein [227].

In summary, the immune responses observed in vaccinated and non-vaccinated piglets were affected by the sow origin, which impacted especially in the cell-mediated immune response, and the natural colonization by a virulent-related strain of *G. parasuis*. F4 protein currently represents a good marker for the detection of

colonization/infection, either by PCR or measuring specific antibodies. We believe that these characteristics make the F4 protein not only a vaccine candidate to consider for future experiments but also an indicator to include for immunity diagnostics of *G. parasuis* in pigs.

Conclusions

F4-adjuvanted formulations with CAF®01 or CDA displayed different immune responses in neonate piglets. Since CAF®01 was able to clear a virulent-related colonizing strain of *G. parasuis* in the nasal cavity in absence of antibodies, CDA elicited an F4-specific systemic response. Circulating specific T-cell populations were influenced by the sow of origin and the colonization status of the animals. A protection study will be needed to evaluate whether the elicited responses by the different combinations can protect against the infection of virulent *G. parasuis*.

Chapter V. Study II.B. Immune and pathological responses after intraperitoneal challenge with virulent *Glaesserella parasuis* in piglets vaccinated with homologous virulence-trimeric associated (VtaA) protein fragment F4 adjuvanted with CAF®01 or CDA

Chapter 5. Study II.B. Immune and pathological responses after intraperitoneal challenge with virulent *Glaesserella parasuis* in piglets vaccinated with homologous virulence-trimeric associated (VtaA) protein fragment F4 adjuvanted with CAF®01 or CDA

Abstract

Traditionally, the efficacy of veterinary vaccines is measured in preclinical studies after the exposure of the animals with the pathogen of interest. In the second part of this study, an intraperitoneal inoculation model was set up to determine the effectivity of experimental *G. parasuis* subunit vaccines based on F4 protein combined with CAF®01 or CDA adjuvants. Immune parameters such specific antibodies in sera, BALF and nasal swabs, as well as pathology were measured in the animals following an intraperitoneal challenge with serovar 5 of *G. parasuis*. Subunit F4 protein vaccines adjuvanted with either CAF®01 or CDA failed in providing protection after the challenge. Piglets developed a severe peritonitis compatible with Glässer's disease without exception that progressed in some animals to other cavities and joints. Clinical signs forced to apply humane endpoints in some cases and finish the experiment four days after the challenge.

Introduction

The main value of a vaccine is its ability to protect against the clinical expression of an infection. Commonly, vaccine efficacy in pre-clinical studies is measured by the reduction of clinical signs (including mortality) or lesions after a controlled challenge with the targeted pathogen. The ideal situation would imply the availability of established immune parameters associated with protection after vaccination, the so-called protection correlates [228]. For *G. parasuis*, protection correlates are not yet established, although it is hypothesized that opsonizing antibodies confer protection against infection [229].

Colostrum deprived piglets are the most commonly used model for the study of *G. parasuis* infection and vaccine efficacy [105,230]. In this model, the lack of pre-existing immunity acquired from the sow via colostrum increases substantially the susceptibility to infectious diseases. Of note, passive immune protection in the pig is acquired from ingested colostrum and milk; therefore, this intake helps to avoid the infection of opportunistic diseases until their immune system is sufficiently mature [231].

Herein, we developed an intraperitoneal *G. parasuis* inoculation model in conventional pigs to reproduce the fibrinous polyserositis and arthritis characteristic of Glasser's disease using 6–7-week-old pigs. This model was subsequently used to evaluate the efficacy of two experimental vaccines consisting of CAF®01 or CDA-adjuvanted F4 protein (described in Chapter IV). For this purpose, we evaluated the compatible clinical signs and lesions of Glässer's disease and the bacterial load of body cavities, joints, and central nervous system (CSF

or meninges). In addition, we determined the specific antibody titres against the vaccine antigen and *G. parasuis* after the intraperitoneal challenge in blood and BALF samples.

Material and methods

Bacterial culture conditions

Strain Nagasaki (serovar 5) of *G. parasuis* was used for the intraperitoneal challenge of piglets. For that purpose, *G. parasuis* Nagasaki was cultured on chocolate agar plates at 37ºC with 5% CO_2 (Biomérieux, Marcy-l'Étoile, France) from a -80ºC stock and overnight growth was resuspended in sterile PBS to reach an optical density of 0.6 measured in a VIS 7200 spectrophotometer (Dinko Instruments, Barcelona, Spain), equivalent to approximately 10^9 CFU/mL. To obtain the working inocula with approximately 10^6 and 10^8 CFU/mL, a 1 to 1000 and a 1 to 10 dilution, respectively, of the original resuspension were performed. After this, inocula were transferred to inoculation bottles and quantified by dilutions and plating.

Ethical regulation

Animal experiments were conducted in AM Animalia (La Vall de Bianya, Girona, Spain) according to the ARRIVE and the Declaration of Helsinki guidelines, approved by the AM Animalia Ethics Committee for Animal Experimentation with reference numbers 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.

Intraperitoneal infection model

Ten Landrace x Duroc piglets (Selecció Batallé, Riudarenes, Girona, Spain) of 6-7 weeks of age were transported to the BSL2 experimental facilities (AM Animalia, La Vall de Bianya, Spain). After a week of acclimation, piglets were weighed, randomized, and split into two groups of 5 animals each. One group was challenged with a total dose of 10^9 CFU and the second with 10^7 CFU of *G. parasuis* Nagasaki strain (Figure 5.1). Inoculation was performed by the injection of 10 mL of bacterial suspension into the caudal right abdomen of the pigs. After challenge, animals were observed daily for clinical evaluation, and humane endpoints were applied when needed (see Clinical signs section). At the end of the study, the surviving piglets were euthanized by an intravenous overdose of sodium pentobarbital (200mg/kg). Necropsies were performed for pathological assessment and *G. parasuis* isolation. Animals were fed *ad libitum* throughout the study. Samples of clotted blood and nasal swabs were collected before the challenge and at necropsy.

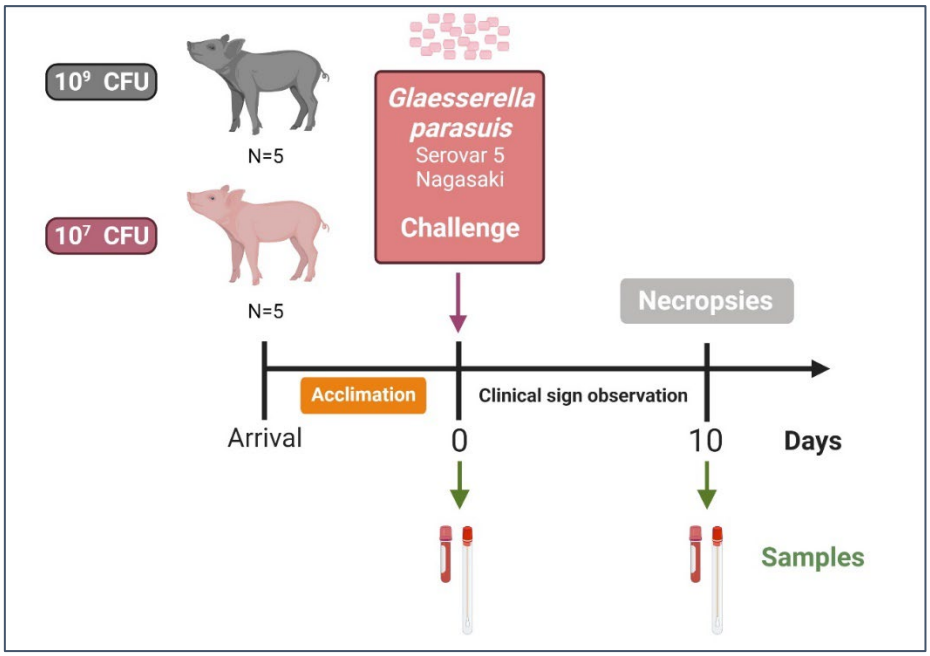


Figure 5.1. Schematic representation of the intraperitoneal challenge study with Glaesserella parasuis. Ten piglets of about 6-7 weeks of age were transported to the experimental facilities and housed during 1 week for acclimation. Then, animals were distributed in two groups of five according to the dose of inoculation. One group received 10^9 CFU, whereas the next group received 10^7 CFU, both by intraperitoneal route. Animals were observed for 10 days for clinical signs. Samples of clotted blood and nasal swabs were collected before the inoculation and on termination day.

Challenge with G. parasuis after vaccination

Piglets from the vaccination study detailed in Chapter IV were challenged with *G. parasuis* 21 days after the second vaccination (D42). Based on the preliminary assessment of the challenge model, 10^9 CFU of *G. parasuis* Nagasaki were intraperitoneally inoculated in each animal as described above. After the inoculation, animals were monitored daily for rectal temperature and specific clinical signs. When required, humane endpoints were applied, and piglets were euthanized by an

intravenous overdose of sodium pentobarbital as described before. The same type of euthanasia was performed at the end of the study with the surviving animals (Figure 5.2). The study was terminated on day four after the challenge. Animals were fed *ad libitum* throughout the study. Blood and nasal swabs samples were collected before the challenge (38 DPV), one day after the inoculation (1 DPI) and at necropsy.

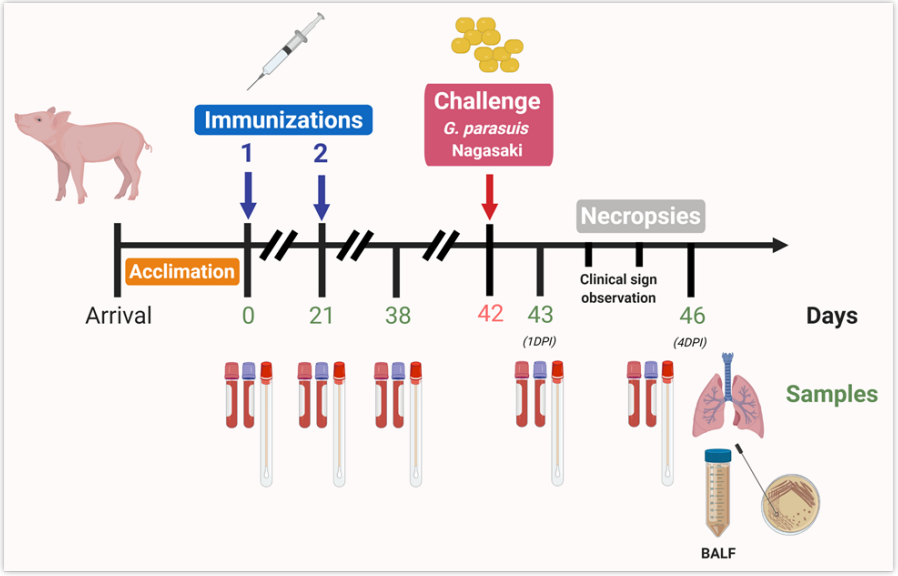
During the post-mortem examination, the presence of *G. parasuis* lesions in body cavities, joints and meninges was evaluated, and samples were taken to assess the presence of the bacterium in the lesions. In addition, a bronchoalveolar lavage (BAL) was performed by filling the right lung of each pig with 150 mL of sterile PBS and recovering 50 mL. Samples of BAL fluids (BALF) were centrifuged at 2,346 rpm (800 x g) for 15 minutes, and supernatants were aliquoted with 1 mM of PMSF and stored at -80ºC until use.

Clinical signs, pathological assessment, and bacterial scores

Rectal temperatures and Glässer's disease compatible clinical signs such as cough, depression, abdominal breath, lameness, joint tumefaction, and nervous symptoms were daily evaluated for each piglet after the intraperitoneal challenge in both studies. Humane endpoints were applied when animals showed rectal temperatures >41ºC (fever was considered above 40°C) or signs of suffering before the end of the experiment. A global score was given for disease severity assessment and endpoint application: 0 = no clinical signs; 1 = one clinical sign observed one day; 2 = observation of 2 clinical signs or one sustained in

time; 3 = severe clinical signs: prostration, nervous symptomatology and/or fever >41°C (euthanasia application).

Glässer's disease compatible gross lesions were assessed at necropsy and scored from 0 to 2 in pericardium, thorax, peritoneum, and four articulations (right and left carpal and tarsal joints), where 0 represents no lesions, 1 mild or limited, 2 extended or generalized lesions. Total score for each animal was obtained from the sum of the obtained scores in pericardium, thorax, peritoneum, and the average of joints. The presence of *G. parasuis* in the lesions was determined by bacterial culture of swabs collected from body cavities (pericardial, thoracic and abdominal cavities), one joint of each limb and meninges or cerebrospinal fluid. Swabs were cultured on chocolate agar by confluent swabbing in half plate and isolation streak in the other half. Plates were then cultured for 24-48h at 37ºC with 5% CO_2. Bacterial scores were given as 1: 1-20 colonies; 2: 21-200 colonies and 3 >200 colonies, using the growth obtained in the first half of the plate. Bacterial growth was confirmed to belong to *G. parasuis* by PCR.



Group	N[1]	Vaccine formulation[2]
1. PBS – NV/C	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.

Figure 5.2. Overall schematic representation of the vaccination study including the intraperitoneal challenge with G. parasuis. Piglets from 3-4 days of age were transported to the experimental facilities and were housed for acclimation for one week. Afterwards, animals were twice immunized on days 0 and 21 of the study. Twenty-one days after the booster, piglets were challenged by the intraperitoneal route with 10^9 CFU of G. parasuis Nagasaki (serovar 5) and monitored for clinical signs. The animal experiment ended 4 days after inoculation.

DNA extraction and G. parasuis detection by PCR

Nasal swabs from sampled timepoints were resuspended in 500 µL of PBS and stored at -80ºC until use. 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. Virulent and non-virulent *G. parasuis* strains were detected by the LS-PCR, which differentially amplifies their *vtaA* leader sequence [104]. Nasal swabs were also tested for serotype-specific PCRs described by Howell et al. (2015), which amplify specific loci of the capsule serovars [207].

Specific G. parasuis and F4 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, the F4 *in-house* ELISA described in Chapter IV was used for the detection of specific anti-F4 total IgG, IgG subtypes 1 and 2, IgM, IgA, and secreted IgA (sIgA) antibodies against F4 in sera, BALF and nasal swab resuspensions.

SP-D detection in BALF by ELISA

Surfactant protein D (SP-D) was detected in BALF by an *in-house* ELISA with a pair of hybridoma supernatants -R-133 (IgG2b) (used as a capture antibody) and R-123 (IgG1) (used for detection)- carried out as previously described with minor modifications [108]. Briefly, high binding 96 well plates were coated overnight at 4ºC with 50 µL of the capture antibody R-133 diluted 1:1 in carbonate bicarbonate buffer. Plates were washed and blocked with blocking solution for 1 h at 37ºC. After an incubation of 50 µL of undiluted BALF sample for 1 h at 37ºC, the plates were washed, and a detection antibody mixture, which

consisted of a half diluted hybridoma supernatant R-123 with blocking buffer, was added to the wells. After incubation for 1 h at 37ºC and washes, ELISA plates were finally incubated for 1 h at 37ºC with an HRP-conjugated goat anti-mouse IgG1 antibody (Invitrogen, Carlsbad, CA, USA) diluted 1:1000. Finally, the reaction was developed with TMB for 5–10 min and stopped with 1N sulfuric acid before reading at 450 nm. Plates were read in a Power Wave XS spectrophotometer (Biotech, Winooski, VT, USA) at 450 nm. SP-D concentrations were finally quantified using a standard curve performed by serial dilutions of recombinant porcine SP-D (U-Protein Express B.V., Utrecht, The Netherlands).

Complement fixation assay

In order to evaluate the ability of the antibodies to attach to their antigen and trigger the union with the complement, a protocol for complement fixation (CFT) was adapted from protocols described before [84,232]. All the reagents used in this assay were firstly titrated using Veronal -barbiturate- buffer (Serovet, Ávila, Spain) as the main diluent. Hyperimmune rabbit serum against sheep RBCs (haemolysin) (Serovet, Ávila, Spain) was used at a dilution of 1:1600 and guinea pig complement (IDVet, Grabels-Montpellier, France) was diluted at 1:40 to achieve 3 haemolytic units (CH_{50}). The challenge strain Nagasaki or *E. coli* BL21 (pASK-IBA2-vtaA9) were used as antigens for the assay at an OD_{600nm} of 1.15 and 1, respectively. Prior to the assay, piglets' sera were heat inactivated at 56ºC for 1h to eliminate complement. Afterwards,

serial diluted sera were mixed with $3CH_{50}$ units of guinea pig complement together with the antigen of interest in round-bottom 96-well plates. After 30 minutes of incubation at 37ºC, a mixture of 3.5% sheep red blood cells previously sensitized with haemolysin was added and further incubated for 30 minutes at 37ºC. After incubation, plates were spun down and supernatants from each well were transferred to a flat-bottomed 96-well plate for measurement at 540 nm in a spectrophotometer. Controls for the haemolysis mixture, complement and antigen were included in each plate. Sample controls of rabbit hyperimmunized serum and pigs from previous studies were also included [55,106]. The absence of haemolysis indicated specific antibody-antigen union and complement fixation to these complexes, while haemolysis indicated an inability to activate the complement system. The percentage of haemolysis in each well was calculated using the following formula: (% haemolysis= OD_{540} test – OD_{540} Blank / OD_{540} total lysis – OD_{540} Blank x 100) [233]. Fixation of the complement to the antibody-antigen complex was considered as the reciprocal dilution obtaining less than 25% of haemolysis.

Statistical analysis

Some variables (Lesion score vs Antibody level Gp) from this study were analysed through the multivariable statistical model described in Chapter IV according to Dohoo et al. (2009) [208]. Graphs and minor descriptive statistical treatments were performed using Prism v9 (GraphPad Software, San Diego, CA, USA).

Results

Infection model with serovar 5 of G. parasuis in conventional pigs

The intraperitoneal administration of the Nagasaki strain of *G. parasuis* resulted in clinical signs one day after inoculation. In fact, one animal inoculated with 10^9 CFU had to be humanely euthanized at that point due to the manifestation of apathy, lameness, and prostration. At necropsy, the piglet had a severe fibrinous peritonitis together with pleuritis and arthritis. In this animal, the challenge strain was isolated from all collected swabs except from cerebrospinal fluid and BALF. The rest of the piglets, although they developed some apathy that lasted two days, remained stable until the end of the study ten days after the challenge. In the last day of necropsies, all pigs inoculated with 10^9 CFU and one piglet from the lower inoculum group (pig number 6) had clumps of fibrin in the abdominal cavity. The rest of the piglets did not show any lesion associated with Glässer's disease, and the challenge strain was only isolated in the BALF from two pigs of each inoculum group.

Nasal colonization assessed by PCR was variable regarding the detection of virulent or non-virulent *G. parasuis* strains before the challenge, but they became mostly positive for virulent *G. parasuis* after the challenge (Table 5.1). Noteworthy, the only pig from the low inoculum group which displayed compatible lesions with Glässer's disease tested negative for virulent strains before the challenge.

Table 5.1. Summary of the results obtained in the infection model study performed with an intraperitoneal inoculation of G. parasuis.

Group	Animal	Termination day	Bacterial score	Nasal colonization[1]		Ingezim Haemophilus Index[2]		F4 ELISA values[3]	
				Before challenge	After challenge	Before Challenge	After challenge	Before Challenge	After challenge
10^9 CFU Higher dose	1	1DPI	17	-	+	Negative	Negative	0.070	0.109
	2	10 DPI	0	-	-	Negative	Doubtful	0.122	2.497
	3	10 DPI	3	+	+	Negative	Doubtful	0.169	2.184
	4	10 DPI	0	-	+	Negative	Negative	0.192	0.356
	5	10 DPI	3	-	+	Negative	Doubtful	0.869	2.256
10^7 CFU Lower dose	6	10 DPI	3	-	+	Negative	Positive	0.398	2.427
	7	10 DPI	0	+	+	Negative	Positive	0.710	2.256
	8	10 DPI	0	+	+	Negative	Doubtful	0.554	2.493
	9	10 DPI	0	+	+	Negative	Negative	0.127	1.113
	10	10 DPI	3	+	+	Negative	Doubtful	1.680	1.881

[1] Virulent G. parasuis detection in nasal swabs by specific PCR. [2] Index of Ingezim-Haemophilus ELISA: <0.4 Negative, 0.4-0.59 Doubtful, ≥0.6 Positive. [3] Expressed as absorbances 450nm.

Sera from the piglets before and after the challenge were analysed to assess the level of antibodies against *G. parasuis*. Before the challenge, all animals tested negative in the Ingezim-Haemophilus ELISA for antibodies against complete *G. parasuis*. After the challenge, the animal that succumbed to the infection tested also negative, while the rest of the animals were mostly positive or at least doubtful in this ELISA test, except for two animals from the lower dose group. In contrast, high variability was observed in the antibody levels against F4 in all piglets. Most of them showed increased levels of F4 antibodies 10 days after the intraperitoneal challenge. The only exception was the animal that died one day after the infection, with the lowest F4-specific antibody levels.

Based on the results from this preliminary experiment, it was decided to use for the subsequent vaccine trial an intraperitoneal challenge with 10^9 CFU of the Nagasaki strain recovered from the diseased piglet number 1.

F4 vaccination did not reduce clinical signs or pathology after intraperitoneal challenge with virulent G. parasuis

A generalized increase in rectal temperatures was observed in all groups with peaks of fever over 41ºC in some animals after the intraperitoneal challenge, without significant differences between groups (Figure 5.3). Severe clinical signs compatible with Glässer's disease, including dyspnoea, apathy, lameness, or neurological signs, appeared in all the groups without exception. These clinical signs, including fever, decreased after the second day post-inoculation in the surviving animals.

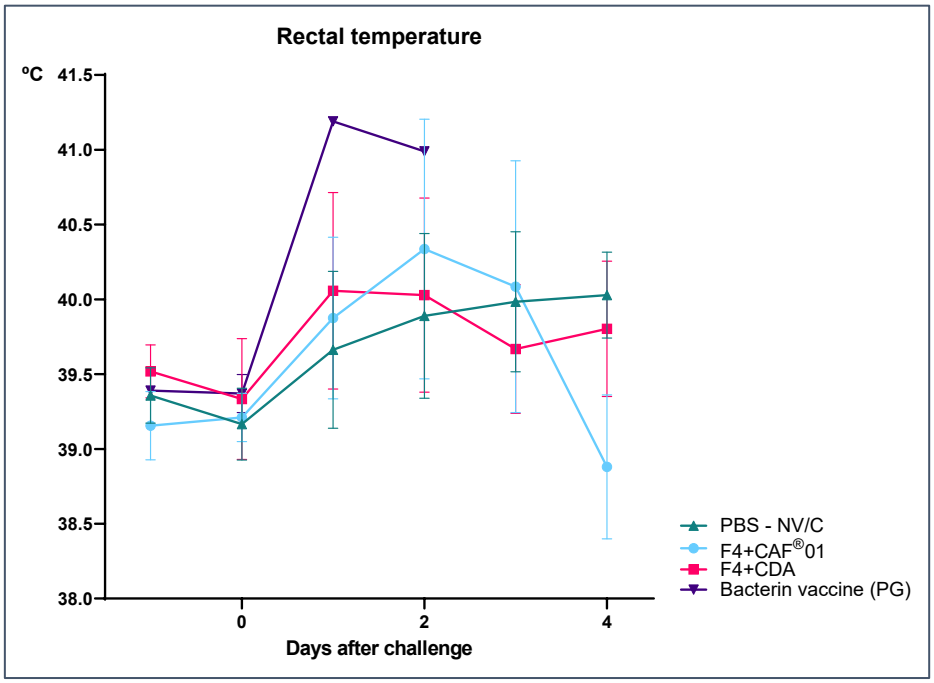


Figure 5.3. Rectal temperature after intraperitoneal challenge with 10^9 CFU of G. parasuis Nagasaki. Piglets were prime-boost vaccinated with F4+CAF®01 (black), F4+CDA (pink), Bacterin (purple) or unvaccinated (turquoise) at 7 and 28 days of age respectively and were challenged 21 days after the booster.

Clinical signs were severe in some cases and included sudden deaths or the need of application of humane endpoints. The two animals vaccinated with the commercial bacterin vaccine succumbed after two days of infection. In addition, 5 out of 7 animals from the CAF®01 vaccine, 3 out of 7 piglets from the CDA vaccine, and 2 out of 6 piglets from the unvaccinated control group needed the application of humane endpoints before 4 DPI. Due to the generalized apathy and poor condition showed by the piglets, 4 DPI was set as the end of the experiment to avoid the unnecessary suffering of the animals (Figure 5.4).

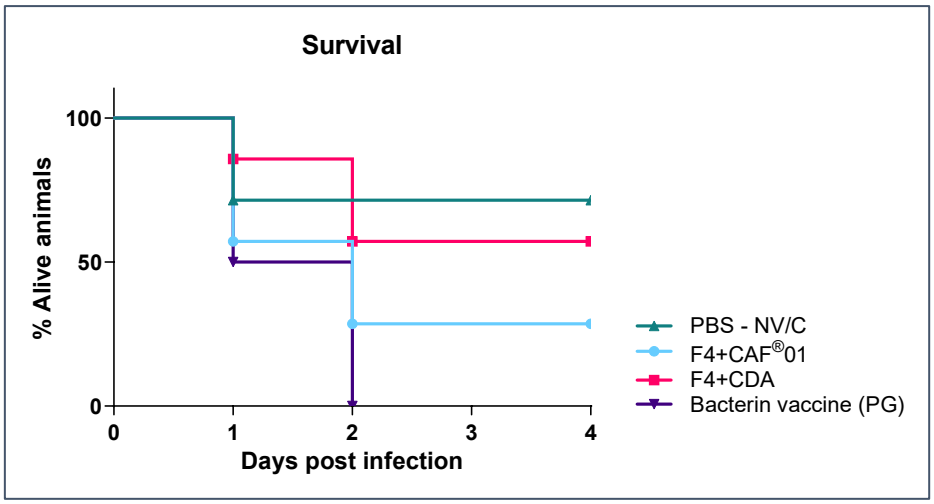


Figure 5.4. Survival curves of the experimental groups after intraperitoneal inoculation of 10^9 CFU of Nagasaki. In coloured lines are depicted: Black (F4+CAF®01), pink (F4+CDA), turquoise green (PBS) and violet (Bacterin group). No significant differences were detected between groups.

Post-mortem examination revealed cases of severe fibrinous peritonitis with ascites, characterized by big patches of fibrin covering the abdominal cavity and packing the intestines, in most of the dead and sacrificed animals in the first and second DPI of all experimental groups. Fibrinous polyserositis was also extended in some cases to the pleura, pericardium, joints, and nervous system. Four days after the inoculation, necropsies revealed in five cases only a milder peritonitis characterized by the presence of residual fibrin patches in the abdominal cavity.

Intraperitoneal challenge affected severely animals from all groups, making it difficult the proper evaluation of the vaccine efficacy. Although not significant, the severity of gross lesions was higher at some timepoints in F4-vaccinated piglets rather than in unvaccinated

ones. Pathology scores changed over time, being more accentuated on day 2 after inoculation than on the rest of the necropsy days. F4+ CAF®01 immunized piglets displayed the maximum lesion score on 2 DPI, whereas the pathology scores from F4+CDA had more variability at 4 DPI (Figure 5.5A). In global, no differences were found between groups (Figure 5.5B).

The multivariable statistical model applied for the evaluation of correlations, showed an association between the anti – G. parasuis antibodies detected in the sow of origin and the lesion score of the animals with a p value of 0.0282, indicating that piglets from negative sows had lower odds to manifest high lesion scores. Post-mortem pictures collected during necropsies depicting the main pathology outcomes are available on appendix (Supplementary table 10).

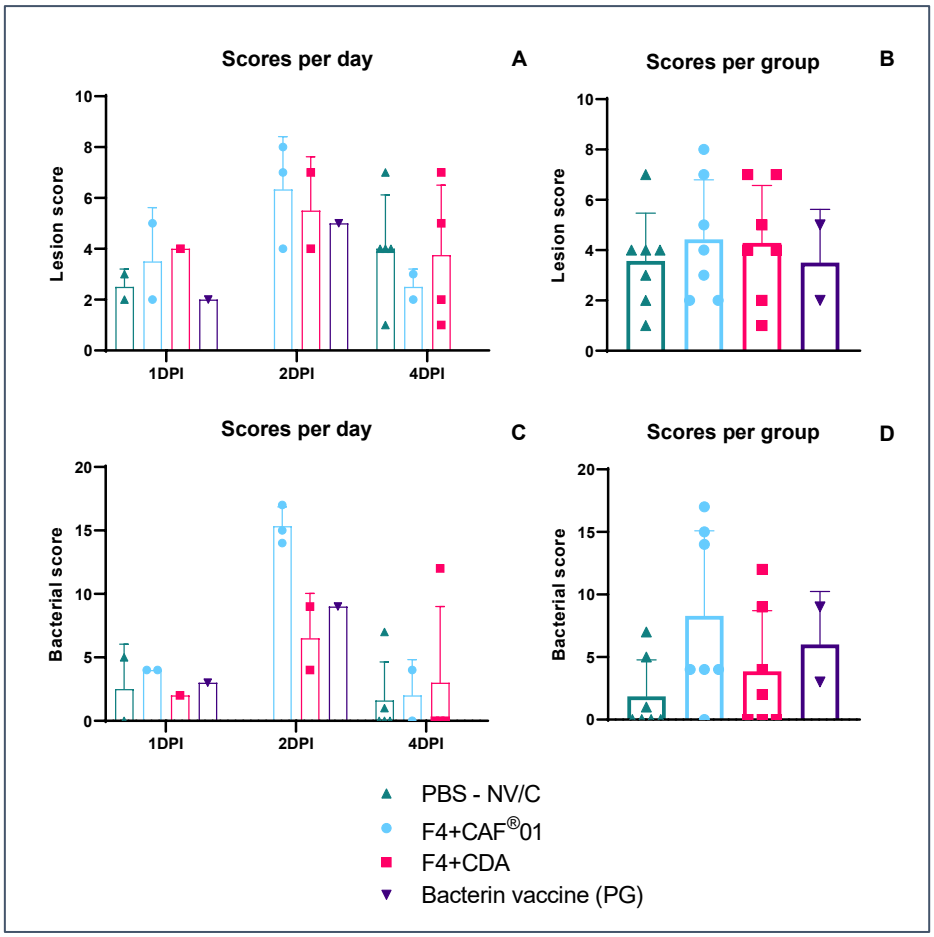


Figure 5.5. Glässer's disease compatible pathology scores represented by day (A) or global per group (B). G. parasuis bacterial re-isolation scores represented by day (C) or global per group (D). All the scores were obtained from the experimentally inoculated animals during post-mortem examination represented in individual symbols. Averages and standard deviations are also plotted.

G. parasuis Nagasaki strain confirmed by ERIC-PCR, was reisolated from most of the swabs collected during post-mortem examination of the animals euthanized on days 1 and 2 DPI. Bacterial scores exhibited a similar trend as pathologic ones, with lower bacterial scores found at day 1, and a peak on day two after the intraperitoneal challenge. F4+

CAF®01 vaccinated animals showed the highest bacterial scores of the study on day two as well as one of the animals vaccinated with Porcilis® Glässer. From the animals that were euthanised at 4 DPI, the Nagasaki strain was recovered in 1 out of 2 F4+ CAF®01 and 1 out of 4 F4+CDA vaccinated animals, whereas it was recovered in 2 out of 5 animals in the unvaccinated control group (Figure 5.5C and Figure 5.5D).

Nasal colonization and F4-specific antibody responses in sera and BALF were altered after the intraperitoneal challenge

Most of the animals with negative or weakly-positive LS-PCR amplification for virulent G. parasuis in nasal samples taken on 38 DPV became strongly positive after the intraperitoneal challenge (Figure 5.6). Two out of 7 animals in both F4+ CAF®01 and F4+CDA vaccinated groups and one out of 7 in the unvaccinated group tested negative for virulent strains after challenge. In fact, these animals remained negative throughout all the study (Supplementary table 5). However, the strong band detected in the PCR from most of the animals was not due directly to the presence of the Nagasaki strain in the nasal cavity, since the serovar 5 PCR was negative in the nose of the piglets. The virulent signal was confirmed to belong to serovar 4 as observed before challenge, during the vaccination section of the study detailed in Chapter IV. Sera collected before and after the challenge were also tested for the detection of G. parasuis by LS-PCR. After one day of the intraperitoneal inoculation only two animals, one vaccinated with F4+ CAF®01 and other from the unvaccinated group, tested positive for virulent G.

parasuis in serum, indicating blood dissemination. These two animals were found dead in their pens without records of fever or any other clinical sign, indicating a death few hours after the intraperitoneal inoculation with serovar 5 of *G. parasuis*.

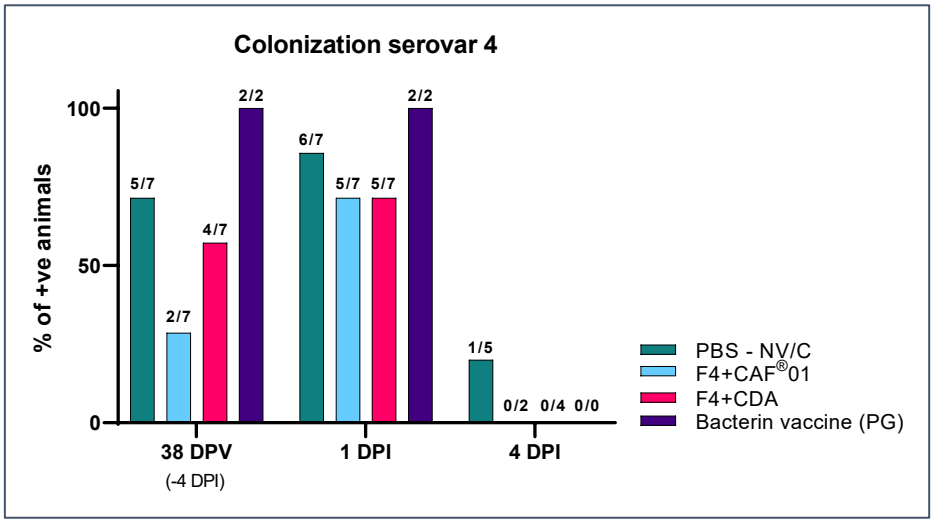


Figure 5.6. Kinetics of nasal colonization of the piglets by serovar 4 G. parasuis during the vaccination experiment measured by LS-PCR from nasal swabs collected before and after the challenge. Bars represent the proportions of positive animals to virulent strains within each group at different timepoints. Above each bar is depicted the number of positive animals out of the total or alive piglets in the case of the last sampling timepoint (4DPI). Positive signals detected by LS-PCR were later confirmed to belong to serovar 4 by G. parasuis serotyping PCR.

A general decrease of F4-specific antibody levels in all analysed isotypes (total IgG, IgG1, IgG2 and IgM) in comparison to the previous sampling timepoint (38 DPV), was observed in the F4+CDA and unvaccinated groups but not with F4+ CAF®01 or Porcilis® Glässer vaccinated animals. In addition, the group immunized with the F4+CDA formulation

maintained a significant higher level of F4-specific total IgG and IgG1 (*P*=0.0174; Figure 5.7A and Figure 5.7B). Similarly, the variability of values stated with IgG2 isotype for every group on day 38 was also retained at 1 DPI (Figure 5.7C). On day 4 DPI, a rise of F4-specific antibody (total IgG, IgG1, IgG2 and IgM) levels was observed in the remaining piglets in both unvaccinated and F4+CDA groups, while this effect was not so apparent in F4+ CAF®01 immunized piglets except for IgM (Figure 5.7D).

Regarding the ability of the antibodies before and after the challenge to activate the complement, the serum from one animal from the Porcilis® Glässer vaccinated group was able to fix complement to the challenge strain with a titre of 8. Furthermore, two animals vaccinated with F4+CDA were able to fix the complement with a titre of 4 against F4-expressing *E. coli*. However, most sera from the piglets remained unable to fix complement to either the Nagasaki strain or the F4-expressing *E. coli* strains.

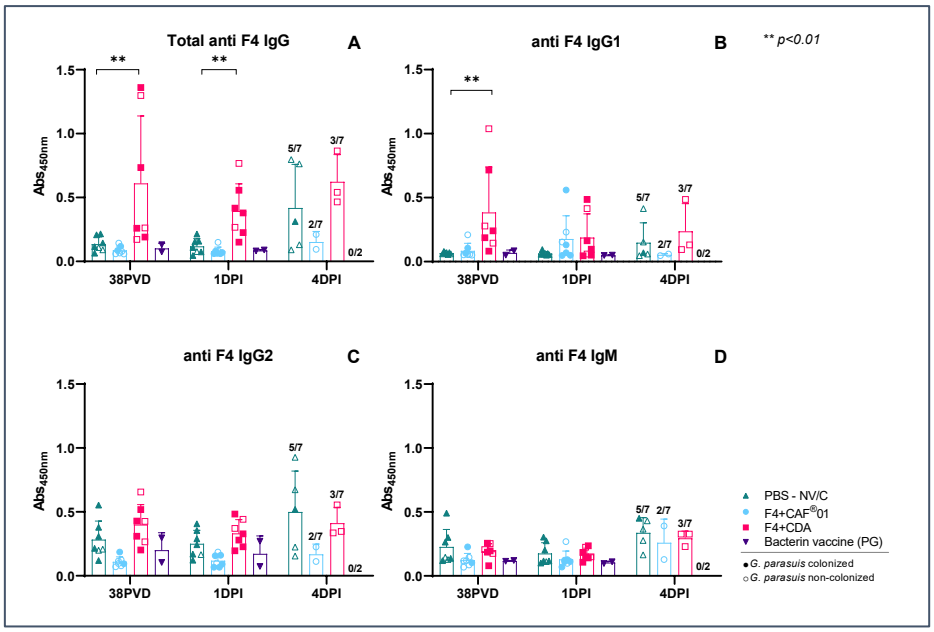


Figure 5.7. F4-specific antibodies total IgG (A), IgG1 (B) IgG2 (C) and IgM (D) detected in sera from pigs immunized with either F4+CDA, F4+CAF®01, PBS or Porcilis® Glässer along the experimental procedure from days 38 DPV, 1 and 4 DPI. Absorbances obtained for each pig are plotted with symbols. G. parasuis serovar 4 colonization is represented with filled symbols, whereas absence of colonization is depicted with empty symbols. Averages and standard deviations of groups are also plotted. On day 4DPI, above each bar are represented the surviving animals of the group out of the total. Statistical significances (*) as well as p values are indicated.

No significant differences were observed between groups in mucosal responses. Nonetheless, some tendencies were noted. All the animals that died or needed to be euthanized on day one after the inoculation had high levels of F4-specific IgG and IgM in BALF ($p<0.05$). This trend seemed to decrease over time, since lower values were observed on days two and four (Figure 5.8A and Figure 5.8B). No differences or trends were seen in total or secreted IgA in BALF, but only a slight increase of the F4-specific secreted IgA in the F4+CDA vaccinated group (Figure

5.8C and Figure 5.8D). On the contrary, SP-D amounts in the lungs increase with the survival time after the challenge ($p<0.05$) (Figure 5.8E).

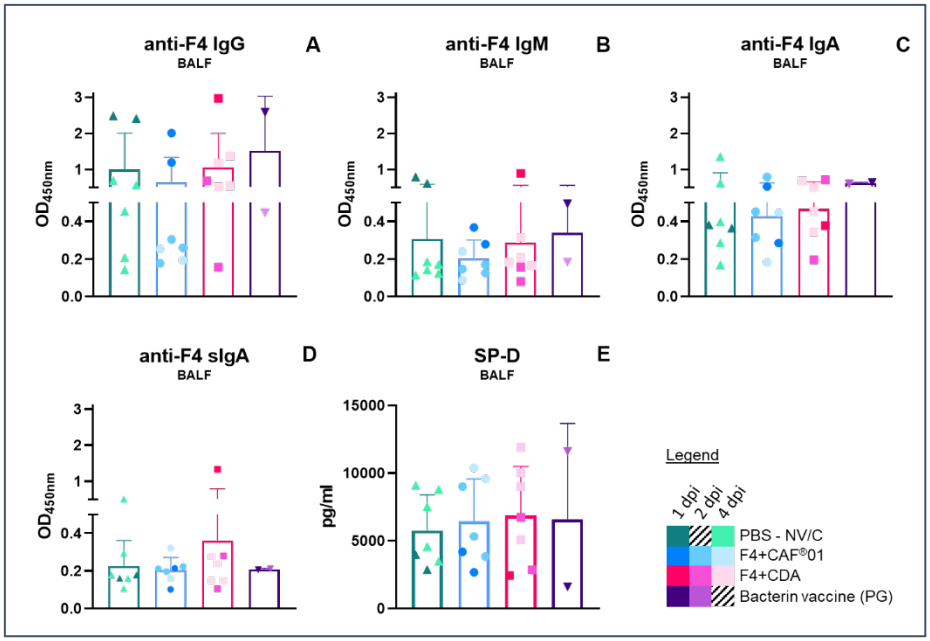


Figure 5.8. Individual humoral responses detected in bronchoalveolar lavage fluid (BALF) were measured through specific in-house ELISAs against F4 protein along with surfactant protein D (SP-D) in BALF. (A) anti-F4 IgG; (B) anti-F4 IgM; (C) anti-F4 IgA; (D) secreted anti-F4 IgA; (E) SP-D levels in BALF. Individual animals are represented in symbols. Colour degradation (from darker to lighter) in symbols indicates the euthanasia days where the samples of BALF were obtained: 1, 2 or 4 days post-inoculation (DPI). Experimental group averages are also plotted (in bars), as well as the standard deviations.

Humoral responses were also evaluated in nasal swabs collected before and after the inoculation. No differences were detected among the groups in specific anti-F4 sIgA prior to the intraperitoneal infection. One day after the challenge, a general increase was observed in the F4-

vaccinated and unvaccinated groups, but not in the two piglets immunized with the bacterin vaccine. This increase was higher in the unvaccinated group, and CAF®01 vaccinated animals showed a significative lower level (Figure 5.9). In each group, the secretion of nasal IgA seemed to be driven by the colonization status of the piglets, since colonized animals showed a stronger secretory response than uncolonized piglets, although none of these responses were statistically significant.

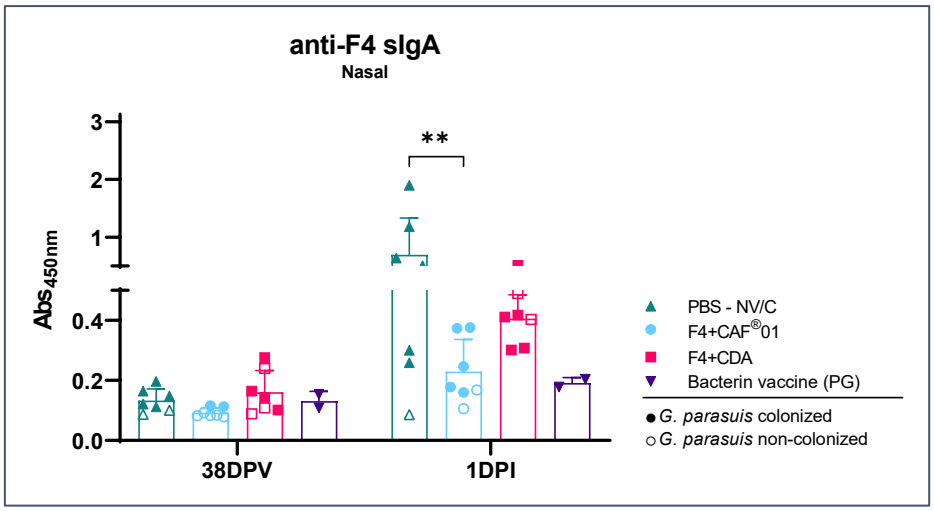


Figure 5.9. Nasal anti-F4 sIgA detected before (38 DPV) and after the intraperitoneal challenge (1DPI). Individual animals are represented in symbols. Experimental group averages are also plotted (in bars), as well as the standard deviations. G. parasuis serovar 4 colonized animals are represented with filled symbols, whereas non-colonized are depicted with empty symbols.

Discussion

After weaning, piglets experience a stressful period due to physiological, immunological, environmental, and behavioural changes that affects severely the health of the animals [34]. In this context, along with waning of maternally derived immunity, piglets can suffer from bacterial diseases caused by different opportunistic pathogens that may cause septicaemia, fibrinous polyserositis and meningitis like *Streptococcus suis, Mycoplasma hyorrhinis* and *Glaesserella parasuis*. Indeed, these microorganisms are considered pathobionts and comprise widespread commensals and strains of diverse degrees of virulence. It is recognized that heterologous colonization is able to confer some cross-protective immunity to the animals [234].

Different factors may be needed to fully reproduce the acute form of the disease and it is not easy to mimic these lesions under experimental conditions using conventional piglets [235]. In this respect, a robust model of experimental infection is still needed for vaccine testing. Here we present two experiments of intraperitoneal challenge, with quite different pathological outcomes, where we could notice that with this type of inoculation the onset of Glässer's disease became advanced in less than 24 hours, a condition seldomly obtained by natural infection route in colostrum-fed animals [62]. In the study of intraperitoneal challenge model we were able to reproduce the same pathological outcomes described by Morozumi and colleagues in 1981 [236]. In terms of antibody levels, most of the animals seroconverted 10 days after challenge except the succumbed piglet, which even had the lowest value of F4-antibodies and was negative for virulent *G. parasuis* colonization

in the nose before the infection. Similarly, in the lower dose group, the only animal that had characteristic lesions of polyserositis, although clotted fibrin, was not colonized by virulent strains. The rest of the 4 animals were colonized before the challenge with virulent *G. parasuis* and they had high antibody values. According to that, even though the inoculum amount influenced the outcome of the disease, previous colonization probably shaped the immune response, as observed in Chapter IV, and may avoid, at least to some extent, the development of the disease as previously reported [237]. All these characteristics confirm that it is easier to reproduce the typical manifestations of Glässer's disease with non-colonized piglets with low antibody titres or lack of them. For this reason, it would have been better to include in the vaccination study only piglets from sows with the low F4-specific antibody levels. Behind this rationale, we assumed with these features the animals might be more susceptible to *G. parasuis* and therefore the typical clinical signs and lesions of Glässer's disease could be reproduced under controlled experimental conditions. However, as explained in Chapter IV, animals from sows with diverse serum levels of specific *G. parasuis* antibodies were included in the study.

Herein we decided to use a high inoculum in the vaccination study to increase the probabilities of producing the typical pathological outcome of Glässer's disease, but seen in retrospect, we believe that a lower CFU inoculum would have been enough to see lesions in seronegative and non-colonized animals. Furthermore, the inoculum prepared for the vaccination study included a Nagasaki strain recovered from a diseased animal. We think that this feature affected the outcome of the disease,

since *in vitro* culture decrease the virulence factors as reported in several bacterial species [238,239]; reversion of this circumstance has also been observed under *in vivo* passages [240].

In the vaccination study, we identified the three described forms of *G. parasuis* disease [7], the hyperacute form in the piglets which died soon after the intraperitoneal inoculation, the acute form in most of the animals and chronic in one pig of the unvaccinated group with consolidated fibrin clumps, furry skin and slow growth which indicated an early infection previous to the challenge. The disease showed a progression with high pathological scores on day 2 and lower ones on day 4 after the inoculation, although it was decided to end the study due to the compromised health of the animals. Lower rates of bacterial re-isolation were observed in piglets vaccinated with the F4+CDA combination. Conversely, the lesion scores in few piglets of this group were similar or even higher at some timepoints comparing to the non-vaccinated animals. Albeit systemic immunization was achieved under F4+CDA or bacterin vaccination with the generation of functional opsonizing antibodies, as it can also be seen with its decrease in almost all the F4-specific and whole *G. parasuis* antibodies respectively, this circumstance did not correlate with a lower disease severity. In addition, CDA or bacterin vaccinated animals that elicited complement-fixing antibodies died on the first day or had higher lesion scores. This fact may contradict the general statement by which extracellular bacterial pathogens are mainly tackled by an effective humoral response, but we cannot rule out an enhancement of the disease as described for some bacterial infections [241]. For example, under

experimental conditions with *Neisseria gonorrheae* in a mouse model, pre-existing antibodies inoculated by passive transfer interfered with the effectivity of vaccine-induced antibodies leading to a delay of complement deposition and increase of bacterial burden [242]. In our case, as mentioned in Chapter IV, piglets suffered from of a milder infection during the vaccination period. The affinity of the vaccine-induced antibodies elicited by the F4+CDA combination is unknown, but it would not be unreasonable to hypothesize that the antibodies generated during the concomitant infection might be interfering with the vaccine-induced ones. In this regard, a thorough study of antibody function and quality deserves further attention to design strategies to avoid this type of undesirable effects.

An increase of virulent *G. parasuis* LS-PCR signal, later confirmed as serovar 4, was detected in the nasal cavity of many of the animals one day after the intraperitoneal challenge. We hypothesized that this increase of a heterologous *G. parasuis* strain in the nasal cavity was an effect of a decreased mucosal immunity produced shortly after the intraperitoneal challenge when the host defences start to tackle the new infection. A similar observation was identified in the study detailed in the next Chapter VI with a rise in the tonsillar burden of *Streptococcus suis* serovar 9 after a lethal intranasal challenge with a heterologous serovar 2 strain.

The parameters measured in lung brought us some issues to remark. Indeed, the piglets that died suddenly or needed the euthanasia application one day after the inoculation had high levels of F4-specific IgG and to a lesser extent of IgM in the BALF. We attributed this

increase in lung antibodies to an increased blood vessel permeability and leakage as a result of the systemic inflammation. A similar increase in the lung vascular permeability associated with the activation and recruitment of neutrophils in the lung was previously reported in surgical peritonitis induced in mice [243]. On the contrary, an inverse relationship can be observed in SP-D secretion in the lung, with lower rates in the animals that died one day after the intraperitoneal infection in comparison to those euthanised later. This effect can be explained again by the general acute inflammation produced in the lung following the intraperitoneal challenge. The loss of the air-blood barrier allows the spill over of SP-D into the bloodstream, with its subsequent reduction in the lung lavage [244,245]. In this regard, SP-D may be used as an indicator of lung damage since the animals sacrificed in the following days had higher concentrations of this analyte in the BALF indicating presumptively a recovery to normal levels.

In the vaccination study, the route of inoculation produced a severe peritonitis generalized in all the piglets without exception. We hypothesize that animals that died on day one might suffered from a septic shock and the rest of the animals that survived manifested a typical Glässer's disease, similar to the previously described peritonitis induced model in pigs with *E. coli* [246]. This type of challenge model bypasses all the natural barriers that pathogens encounter under natural infection conditions. In the case of intranasal inoculation, the fibrinous polyserositis of Glässer's disease is the result of a severe inflammation caused by septicaemia after the multiplication of virulent *G. parasuis* in the lung [247]. In our experiment, we induced fibrinous peritonitis by

directly injecting the bacteria into the abdominal cavity, which led to a vasodilatation that increased the permeability of vessels with exudates of fluid rich in complement, clotting factors, fibrinogen and antibodies [248]. We presume this vasodilatation was exploited by the bacteria to invade the bloodstream and produce septicaemia, enabling an inverse pathway to that which occurs during a natural infection. This fact can be asserted as well by the presence of the challenge strain in the joints and meninges of some animals in the study. However, fibrin deposits in body cavities not only are the pathologic result of the severe inflammation but are also considered a mechanism of host defence [249]. Thus, fibrin deposits constitute a scaffold by which the bacteria can be entrapped and cleared by the large peritoneal macrophages, produced in the milky spots from the omentum [250]. This characteristic pathologic reaction of Glasser's disease was revealed at post-mortem examination in most of the animals vaccinated with F4+CDA formulation with massive fibrin deposits inside their abdominal cavities. Unlike the rest of the groups, CDA animals elicited a stronger specific antibody response. Both characteristics deserve especial attention in our opinion since this adverse reaction might be attributable to some sort of antibody enhanced disease (ADE) as exposed above.

On the other hand, both studies reported in this chapter helped us to identify the potentiality of the F4-specific ELISA, which not only detects antibodies with high accuracy but it also can detect previous colonization. This feature contrasted to the Ingezim Haemophilus kit, where the high threshold by which measures antibody levels in sera

(low sensitivity), impeded a correct evaluation of pre-existing low-level immunity.

In Chapter IV we detected humoral responses and changes in the nasal colonization by serovar 4 *G. parasuis* in the vaccinated piglets, indicative of a partial immunization using F4+CDA and protection against a natural colonization in the F4+CAF®01 combinations, respectively. Nonetheless, both responses revealed to be insufficient to protect the animals against the intraperitoneal challenge. The high severity of the challenge, together with the limited number and type of outbreed piglets, dampened the obtention of homogeneous responses and significant results. As there are no established immune correlates of protection for Glässer's disease vaccines, the measure for protection relies mainly on the survival and pathological assessment after the challenge. For this reason, we believe further research will be needed to evaluate the immune correlates of protection, especially for those systemic bacterial disorders in pigs including Glässer's disease. This knowledge may help to improve the tests needed for the assessment of vaccine efficacy and will be invaluable for the design of effective vaccines, including those directed to immature neonates.

Conclusions

F4-protein combinations adjuvanted with either CAF®01 or CDA failed in providing protection against an intraperitoneal challenge with serovar 5 of *G. parasuis*. Albeit both formulations elicited different immune responses after the challenge, the quantity, nature, and route of infection was excessive to extract conclusive results regarding the potential protective abilities of both combinations.

Chapter VI. Study III. Immune responses in new-born piglets after immunization with *Streptococcus suis* surface-conserved antigens combined with CAF®01 or CDA

Chapter 6. Study III. Immune responses in new-born piglets after immunization with *Streptococcus suis* surface-conserved antigens combined with CAF®01 or CDA

Abstract

Streptococcus suis, a gram-positive bacterium from the *Streptococcaceae* family, colonizes the upper respiratory tract of piglets at early stages of life. However, virulent serovars of *S. suis* may develop a systemic infection characterized by septicaemia, polyserositis and meningitis which may cause high morbidity and economic losses to the swine industry. The treatment of streptococcal disease relies basically in good husbandry of the herds and antimicrobials. For this reason, there is a need of effective and alternative tools to fight the disease albeit the vaccines available against are based mainly in autogenous vaccines of farm level. In this study, we aimed to test the immunogenicity and effectiveness of the conserved immunogens MRP2 and C05 derived from surface exposed proteins of the cell wall of S. suis, adjuvanted with CAF®01 and CDA in newborn piglets. Thirty-two neonate piglets were distributed into five groups, including adjuvanted groups, non-adjuvanted and unvaccinated control, besides a group immunized with Bacterin adjuvanted with Specol®. Animals were twice immunized from their second day-of-life in an interval of 21 days. On day 50, piglets were intranasally challenged with 10^9 CFU of serovar 2 from *S. suis* and left for clinical observation. Samples of blood and tonsillar swabs were collected throughout the study to assess humoral, cell-mediated

immune responses and bacterial burden. Subunit vaccinated animals elicited a weak immune response either humoral and cell-mediated that correlated with low protection and mortality after intranasal challenge. On the contrary, most of the piglets immunized with the autogenous vaccine became protected after the challenge with a detectable humoral response. Although MRP2+C05 vaccines failed in providing a protective immune response, this study contributes with new knowledge in how to formulate further vaccine combinations against streptococcal disease.

Introduction

Streptococcus suis (S.suis) is a chain-forming coccus-shaped Gram-positive bacterium from the S*treptococcaceae* family. *S. suis* is considered a pathobiont that naturally colonizes the upper respiratory tract of pigs and wild boars, especially the tonsil and the nasal cavity. *S. suis* comprises 29 serovars and some of them can be pathogenic for pigs with zoonotic potential, such as serovar 2 [64,251]. Swine streptococcal disease can affect pigs of all ages, but nursery piglets are especially susceptible to disease due to a combination of stress and decreased of maternally derived immunity, which affects the immune status of the piglets. Severely clinically affected piglets show pyrexia followed by sudden death and/or, nervous signs and lameness. Pathologically, *S. suis* associated disease is mainly characterized by fibrin deposits on internal organs (polyserositis, including meningitis) and in joints (polyarthritis) [67]. *S. suis* is responsible for a considerable economic burden in the pig industry worldwide. Outbreaks appear regularly,

with occasional transmission to humans in several developing countries, which makes *S. suis* a pathogen of zoonotic concern [66,252]. The control of *S. suis* mainly relies on good animal husbandry combined with strict biosecurity protocols on farms. *S. suis* infection can only be treated nowadays with antibiotics either to individual sick pigs or metaphylactic use. Nevertheless, the misuse of antibiotics in livestock have led to increasing emergence of antimicrobial resistances and therefore the use of these compounds is restricted in many European countries [253].

Neonates and young piglets are vulnerable to infectious diseases like the one caused by *S. suis*, implying severe welfare problems and economic losses. In this context, vaccines have become the most important strategy to control and protect against early-life infections. Vaccination in the first week of life leading to the generation of an early immune response could reduce disease and mortality around weaning [254]. However, the immaturity of the neonate immune often display a less effective response after vaccination skewed towards Th2 and Treg responses [31,32]. Current *S.suis* vaccines are mostly autogenous preparations from the bacterium isolated at the individual farm level. In this regard, a commercial vaccine that may provide cross-protection against a wide range of virulent serovars is highly needed. Vaccine immunogens generated from conserved epitopes present in surface proteins involved in virulence have become an interesting approach [86]. However, subunit proteins often lack an intrinsic activity capable of triggering an adequate immune response. Hence, this type of vaccine formulations need to include adjuvants capable to target the

requirements of the neonatal immune system and contributing to an increased vaccine efficacy [202].

"Muramidase-released-protein" (MRP) is a 136 KDa cell-wall anchored protein considered one of the main virulence markers in *S. suis* [109], whose binding functions to several host proteins remain to be completely elucidated [255]. MRP adjuvanted with water-in-oil emulsion Specol® induced protection against challenge with *S. suis* serotype 2 [112]. In this study, we will use as immunogens a surface exposed and conserved sequence derived from MRP called "MRP2" with the C05 antigen. C05 is a fragment of the cell-wall protein tagatose-6-phosphate aldose/ketose isomerase which is involved in the metabolism of amino sugars. C05 has been recently studied (SSU0185) in mice and pigs providing protective immune responses against a challenge with *S. suis* along with other 4 subunit proteins [114,115].

Herein, in the present vaccination study, we aimed to determine the efficacy and immunogenicity of MRP2 together with C05 antigens combined with either "Cationic Formulated 01" (CAF®01) or bis-(3,5)-cyclic dimeric adenosine monophosphate (CDA) in new-born pigs. CAF®01 is a liposome-based adjuvant composed of N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) and α,α'-trehalose 6,6'-dibehenate (TDB), a Mincle agonist glycolipid analogue to the cord factor of *Mycobacterium tuberculosis* that induce Th1/Th17 cell-mediated besides humoral immune responses [131]. CDA is a cyclic dinucleotide, firstly described in *Listeria monocytogenes* and known to activate TNF and type I Interferon (IFN), stimulating Th1/Th2/Th17, cytotoxic and humoral

immune responses through the Stimulator of Interferon Genes (STING) [146]. Moreover, to better determine the immunogenicity and the putative effectivity of both combinations, a positive control vaccine composed of formalin-inactivated bacterin of *S. suis* serotype 2 emulsified with Specol® was included in the study. This vaccine formulation has shown efficacy in a previous study after a homologous challenge with *S. suis* [256].

Material and methods

Bacterium culture and bacterin preparation

The *S. suis* strain S10 (SS10) from serotype 2 was used in this study. Recovering cultures of the bacterium were mainly performed in Columbia Sheep Blood agar (WBVR, Lelystad, The Netherlands) for 18h at 37ºC.

SS10 strain was formalin-inactivated for bacterin preparation, used for different purposes throughout the study. The strain was recovered from -80ºC in Columbia blood agar overnight at 37ºC. A previous culture in Todd-Hewitt Broth (THB) (WBVR, Lelystad, The Netherlands) with a single colony was performed overnight at 37ºC without shaking. After the pre-starter propagation, cultures were grown in THB to reach the desired concentration of 10^9 CFU/mL. Harvested growth was washed with PBS and inactivated with 0.5% formalin for 1 h at room temperature (RT) with intermittent gentle mixing. Afterwards,

inactivated bacterial suspension was washed, resuspended in PBS, and maintained at 4ºC until use.

Propagation of the bacteria for inoculum was performed in three steps. One recovery step from -80ºC, culturing overnight the bacterium in Columbia Sheep Blood agar for 18h at 37ºC, followed by a second pre-starter step culture in THB for 18 h at 37ºC. The volume of the pre-starter culture was used to achieve the bacterial growth in the log phase (third step) to reach an OD_{600nm} of 0.5 measured by spectrophotometry. After adjustment, cultures were washed twice with PBS to clean the bacteria and finally resuspended in PBS to reach an OD_{600nm} of 1±0.02 (approximately 10^8 CFU).

Antigens and adjuvants, vaccine preparation

MRP2 and C05 antigens from *S. suis* were synthesized and provided by Dr. J.M. Wells (Wageningen University, The Netherlands).

CAF®01 and CDA adjuvants were provided by the *Statens Serum Institut* (Copenhagen, Denmark) and *Helmholtz Zentrum for Infektionsforschung* (Braunschweig, Germany), respectively. The CAF®01 mixture was prepared 1:1 (v:v), diluting the antigen with Tris Buffer to reach one mL per dose. CDA vaccine formulation was prepared admixing immunogens and adjuvant in PBS to reach one mL per dose (Table 6.1).

For homologous bacterin formulation, SS10 bacterin was emulsified with water-in-oil Specol® immunogenic adjuvant (Wageningen Bioveterinary Research (WBVR), Lelystad, The Netherlands) in a

mixture ratio of 1:1. Each animal was primed and boosted with 1 mL of the vaccine in the right and left hind legs respectively.

Table 6.1. Distribution of the experimental groups with the corresponding vaccine formulations.

Group	N	Antigen	Adjuvant
1. *PBS - NV/C*	4	PBS	PBS
2. *MRP2-C05*	5	MRP2 (50 µg) + C05 (50 µg)	None
3. *MRP2-C05/CAF®01*	8	MRP2 (50 µg) + C05 (50 µg)	CAF®01 (0.5 mL)
4. *MRP2-C05/CDA*	7	MRP2 (50 µg) + C05 (50 µg)	CDA (30 µg)
5. *SS10/Specol® (Bacterin)*	8	4 x 10^8 CFU inactivated SS10	Specol® (0.55 mL)

Formulations expressed per dose in a final volume per animal of 1 mL, injected in each hind leg.

Experimental design and animal housing

Two-day-old Topigs Norsvin Z-line, commercial breed pigs (Van Beek SPF Varkens B.V., Putten, The Netherlands) from different sows of parity between 2 and 5, were taken from their litters and transported into the experimental BSL1 facilities at Wageningen Bioveterinary Research (Lelystad, The Netherlands). After three days of acclimation, the piglets were randomized into four pens according to their weight and the sow origin. Animals from groups one and two were allocated in one pen, whereas the rest of the groups were placed in different pens. Animals were prime vaccinated following the groups represented in Table 6.1. Twenty-eight days later, the piglets were boosted and left for 22 days before the challenge. One week before the challenge, animals were transported to the vetBSL2 facilities of the WBVR for acclimation.

Groups one and two were housed in one room, the remaining animals were acclimated in one room per group. On the day of challenge held 22 days after the booster, the nasal mucosa of the animals was sensitized prior to the infection with a 1% acetic acid solution applied with a mucosal atomization device [257]. After 3 hours, the animals were sedated with a mixture of Zoletil® 4 mg/kg (Virbac Nederland BV, Barneveld, The Netherlands) and Sedanum® 2 mg/kg (Dechra Veterinary Products BV, Amsterdam, The Netherlands) and challenged by intranasal route with 10^9 CFU of strain SS10 of ST2 of *S. suis* in a volume of 1.5 mL in each nostril. Animals were left for clinical sign observation for a maximum period of 8 days. The piglets were sampled during the study in several timepoints for clotted blood, heparin blood and tonsillar swabs (Figure 6.1). Piglets were fed *ad libitum* during all the experiment according to standard nutrition plan for colostrum-reared animals. Piglets were colostrum-fed (2-3 days before the experiment), milk-fed four times a day (until D6), mash-fed (until D21), afterwards the animals were nourished with pellets (Denkavit Nederland B.V., Voorthuizen, The Netherlands). Pens were enriched with toys for the development of the natural behaviour of neonate piglets. The animal experiment was conducted in accordance with the Dutch animal experimental and ethical requirements and the project license application was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD), permit number AVD40100202010304.

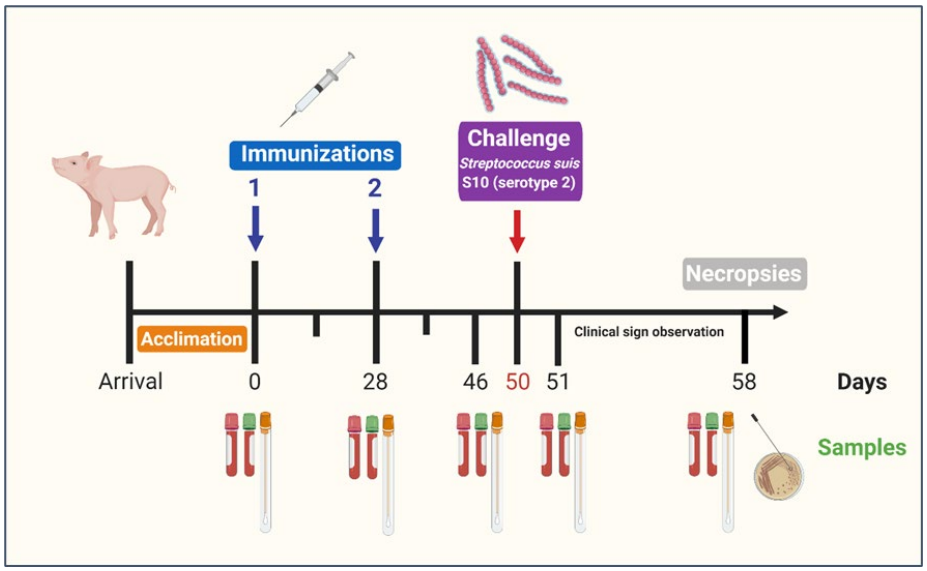


Figure 6.1. Schematic representation of the study. Piglets of 2-3 days of age were transported to the experimental facilities and left 2 days for acclimation. Animals were immunized on day 0 and sampled on day 1 for blood. After 4 weeks, the piglets were boosted (D28) and sampled for blood and swabs on days 28, 37 and 46. The lethal challenge with Streptococcus suis S10 strain (SS10) was performed by intranasal route on day 50. Animals were maintained for clinical evaluation and possible necropsies for 8 days until the end of the experiment. Created with BioRender.com.

Clinical sign evaluation

Animals were evaluated daily for unspecific signs before the challenge; weight and rectal temperatures were daily monitored during the experiment. After the infection, piglets were examined thrice a day for *S. suis* related clinical signs such as fever, tremors, apathy, lameness, and neurologic signs. Humane End Points (HEP) were immediately applied once the diseased animal was not willing to move, stand on three legs or manifested central nervous clinical signs. Prior to sacrifice the pigs were sedated as above described and then were euthanized by

an overdose injection of pentobarbital (Euthasol 40%, AST Farma, Oudewater, Netherlands) in the auricular vein according to standard procedures.

Bacteriological and pathological assessments

After the challenge, animals were euthanized with an overdose of sodium barbiturate applying HEP when required according to the standard procedure mentioned above. After euthanasia, animals were exsanguinated and subjected to necropsy and samples were taken for subsequent gross-morphology assessment and bacterial evaluation. Body cavities: abdomen, thorax and pericardium and joints -shoulders, elbows, carpus, knees, and tarsus-, were aseptically dissected and thoroughly observed for fibrinous polyserositis and arthritis evaluation and swabbed for bacterial isolation. The cranium was also explored for meningitis gross evaluation; samples of cerebrospinal fluid or meningeal swabs were collected. Pathology scores were given according to the severity of the observed gross lesions: 0 absence, 1 mild, 2 moderate and 3 severe; total score is obtained from the sum of the scores given to each body cavity, joints, and cranium. All the swabs were streak-cultured in Columbia sheep blood agar and incubated overnight at 37ºC for *Streptococcus* presence. After incubation, bacterial scores were given as follows: 0 absence, 1 for 1-20 colonies, 2 for 20-200 colonies, 3 >200 colonies; likewise, total bacterial scores were obtained from the sum of all swabs. Alpha-haemolytic colonies were selected and picked for identification and confirmation by MALDI-TOF with a

MALDI Biotyper Microflex V.3.1 (Bruker Nederland BV, Leiderdorp, The Netherlands) using the Bruker taxonomy database.

DNA isolation and tonsillar cps2 and cps9 S. suis detection by qPCR

DNA was isolated from tonsil swabs as follows. Flocked ESwab™ with Amies medium (Copan Diagnostics, Murrieta, CA, USA) tips collected throughout the study were aseptically cut off and incubated with 2 mL PBS, vortexed and treated by sonication at 40 Hz for 1.5 h on ice. Prior to DNA isolation, 154 µL of swab sample samples were treated with an enzyme mix to lyse the bacterial cells (46 µL of lysis mixture containing 20 µL lysozyme (100mg/mL), 1 µL mutanolysin (5000 U/ml) and 25 µL Protein kinase K 600 AU/mL, included in DNeasy Blood & tissue kit (QIAGEN Benelux B.V., Venlo, The Netherlands). Samples were mixed by vortex and incubated for 30 min at 37°C. Two hundred µL of AL buffer from the Qiagen Blood and tissue kit was added, and samples were then vortexed for 15 seconds and incubated for 30 min at 56°C. Two hundred µL of absolute ethanol was added and the samples were vortexed for 15 sec. Purification was continued from step 4 of the Qiagen DNeasy Blood & tissue kit manual. Purified DNA was eluted in 30 µL SuperQ® water. DNA concentration was measured using a NanoDrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Bleiswijk, The Netherlands) and Agilent Technologies 2200 TapeStation and a BMG Labtech CLARIOstar.

Primers and probe sequences specific for the cpsJ locus of S. suis serotype 2 (cps2J) and the cpsH locus of S. suis serotype 9 (cps9H) have

been previously described [258]. Internal positive controls (IPC) for the cps2J PCR and the cps9H PCR consist of a DNA fragment in a pUC57 plasmid with primer binding sites for cps2J or cps9H and a probe binding site (Table 6.2). The regions in between the primer and probe binding sites are filled up with random DNA sequence with the same length as the PCR fragment ensuring no specific binding takes place. Primers and probes were produced by Biolegio (Nijmegen, The Netherlands), and the IPCs were made by Genscript Biotech (Rijswick, The Netherlands).

For each *cps2J* PCR or *csp9H* PCR a standard curve control containing $2.5x10^{-1}$ - 10^{-7} ng genomic DNA of *S. suis* serotype 2 strain SS10 or *S. suis* serotype 9 strain 8067 was added, respectively. A standard curve control for the IPC was also included using $1x10^{-4}$- 10^{-8} ng pUC57-*cps2J* DNA or pUC57- *cps9H* DNA. The slope for the standard curves should lie between -3.1 and -3.5. Negative controls containing no DNA were also included. Each reaction with a final volume of 20 µL contained 10 µL 2X Taqman Fast Universal PCR mix (Thermo Fisher Scientific, Bleiswijk, The Netherlands), 1.8 µL 10 pmol forward primer (F-cps2J or F-cps9H), 1.8 µL 10 pmol reverse primer (R-cps2J or R-cps9H), 0,25µL test probe (FAM-cps 2J or FAM-cps9H), 0.25µL IPC probe (VIC-IPC_cps2J/cps9H). To the experimental samples, 2.5 µL of DNA isolated from tonsil swabs was added. 1 µL IPC DNA ($1x10^{-7}$ ng/reaction of pUC57 -*cps2J* or pUC57- *csp9H*) was added to all samples except the IPC standard curve samples. The reactions were added up to 20 µL with SuperQ® water (Merck Millipore, Amsterdam, The Netherlands). PCRs were performed on ABI 7500 FAST real-time PCR systems, Applied

Biosystems. PCR conditions: 5 min 95°C, 40X [15 sec 95 °C, 1 min 60°C], probe detection FAM/VIC, QPCR cut-off 0.1. The amplification curves were analysed with the ABI 7500 2.3 software of Applied Biosystems (Waltham, MA, USA). The uninhibited Ct for $1x10^{-7}$ ng pUC57- *cps2J* DNA and pUC57- *cps9H* DNA lies between 30-31 in both PCRs.

Table 6.2. Primer, probes, and controls of the qPCR used for S. suis quantification in this study.

Name	Sequence 5'-3'	Reference
Primers		
F-cps2J	ACGCAGAGCAAGATGGTAGAATAA	Dekker et al. 2016 [258]
R-cps2J	TGCCGTCAACAATATCATCAGAA	
FAM-cps 2J	CAAACGCAAGGAATTACGGTATC	
F-cps9H	CAAAGTTAGTTCAGGAAGGAATAGTCT	
R-cps9H	CCGAAGTATCTGGGCTACTG	
FAM-cps9H	6FAM-TTTCAGATCAAGATGATATTTGGGACT-BHQ	
Internal positive control probe and primer sequences (IPC)		
VIC-IPC_cps2J/cps9H	VIC-AGCTTTCGAGTCGGGC-BHQ	This study
IPC fragment cps2J in pUC57	ACCCAGAGCAAGATGGTAGAATAAGCA TCCTTAAGGCGTTAAAAGCTTTCGAGTC GGGCGTAGCTAGAAGGCTTTTGGGGAG TCGTACTGACGTGCATGCATGGCCTAAC CTTCTGAATG ATATTGTTGACGGCA	
IPC fragment cps9H in pUC57	CAAAGTTAGTTCAGGAAGGAATAGTCT CGGATTAGCTGACTGCTTCAGGCATGCA TGGCATCCTTAAGGCGTTAAAAGCTTTC GAGTCGGGCGTAGCTAGAAGGCTTTTGG GGAGTCGTACTGACGTGCATGCATGGCC TAACCGTGGACCTGCATCAGTAGCCCA GATACTTCGG	

Specific antibody response - ELISA tests

Clotted blood tubes were centrifuged at 2500 rpm for 10 minutes to obtain serum. Serum samples were aliquoted and stored at -20ºC until use. Greiner MICROLON®600 high binding ELISA plates were coated overnight at RT with approx. 10^6 CFU/well inactivated *S. suis* serotype 2 strain SS10 in PBS or with the subunit antigens MRP2 (250 ng/well) or C05 (125 ng/well) in carbonate buffer. Blocking was performed with PBS + 1% BSA at pH7.2 for 1 h at RT. Sera from piglets were diluted at 1:100 and 1:300 in PBS and added to the wells for complete bacteria and 1:100, 1:500 and 1:2000 for subunit antigens. Bound antibodies were detected with a 1:10.000 dilution of peroxidase (PO) - conjugated anti-porcine-IgL (mouse antibody (MAb) clone 27.2.1; Sinkora et al., 2001 [259] (WBVR, Lelystad, The Netherlands)) using tetramethylbenzidine (TMB) as a substrate. Reactions were stopped after 10-15 min by addition of 1N H_2SO_4 and extinctions (450 nm) were measured on a Microplate Reader. Serum and secondary antibody incubations were performed for 1 h at RT and wells were washed three times with PBS after both incubations. Sera from a surviving pig that had suffered an infection with *S. suis* serotype 2 strain SS10 and a caesarean-derived colostrum-deprived (CDCD) piglet from previous studies were used as positive and negative controls respectively. A dilution series of the positive control was measured in duplicate, and a standard curve was fitted using 4-parameter logistics with SoftMax Pro Software. The standard curve was used to interpolate OD_{450nm} values of individual samples to concentrations relative to the positive control ($\%_{pos}$). All sera were analysed in duplicate. Optimal dilutions of the coating antibodies, the

matrices, the conjugates, and the positive internal control sera were determined during preliminary standardizations.

Opsonophagocytosis and killing assay (OPKA) with porcine neutrophils

Neutrophils (PMN) from two healthy piglets of 6-8 weeks of age were isolated from heparin blood tubes through a Ficoll-Paque™ PLUS 1.077 g/mL (GE Healthcare, Eindhoven, The Netherlands) gradient. After centrifugation, supernatants, buffy coats and Ficoll layer were carefully discarded. The red pellets were then treated by osmotic shock until the complete removal of the erythrocytes. PMN concentration was adjusted to 5×10^6 cells/mL. Complement source was obtained by pooling porcine normal serum from 3 healthy animals. Sera from experimental piglets collected on day 46 were previously heat-inactivated by 30 min at 56ºC for complement inactivation.

The OPKA assay consists of two steps, opsonization and killing with PMN. For the first step, a mixture of complement source, test sera and bacteria in a MOI of 0.3 was incubated for triplicate in round-bottomed 96-well plates for 15 min at 37ºC. The second step begin after the opsonization when the volume containing PMN cells was added to the mixture for phagocytosis. Plates were then incubated in shaking for 1 h at 37ºC in 5% CO_2 atmosphere. Bacterial survival was calculated by comparing bacterial counts (obtained by serial dilutions and plating) before and after phagocytosis incubation. The assay was performed twice with PMN from two different animals of the same age.

Immune phenotyping of circulating and stimulated PBMCs by flow cytometry analysis

Heparin tubes collected from the jugular vein of each animal were first incubated in rotation at RT for 30 min. Two hundred microlitres were taken for general haematology analysis using a POCH-100iV-diff Haematology analyser (Sysmex Nederland BV, Etten-Leur, The Netherlands). For the analysis of circulating PBMCs, 200µL of uncoagulated blood was also transferred to 96-well cell culture plates and lysed with ACK lysis buffer. After two washes, cells were stained with a mixture of surface markers as described below (Table 6.3).

The rest of the blood was processed for PBMC isolation diluting the blood volume 1:1 in PBS and transferring to Leucosep® tubes (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) using a Ficoll-Paque™ PLUS 1.077 g/ml (GE Healthcare, Eindhoven, The Netherlands) gradient. Buffy coats were treated with ACK Red Blood cell lysing buffer (Gibco® Thermo Fisher Scientific, Bleiswijk, The Netherlands) to remove remaining erythrocytes. After that, isolated cells were counted with a Particle counter Z2 (Beckman Coulter Nederland B.V., Woerden, The Netherlands) and adjusted to the desired concentration.

Flat-bottomed 96-well cell culture plates (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) were prepared for stimulation and subsequent immunophenotyping for the detection of memory/specific activated T and Th responses. For that purpose, $5x10^5$ cells per well were incubated for 48 h at 37 °C 5% CO_2 under the presence of 5 µg/mL of Concanavalin A (Sigma-Aldrich, Madrid, Spain) as a positive control,

sole medium as a negative control, 2 μg/mL of MRP2 protein or inactivated *S. suis* challenge strain SS10 in an MOI of 10. After incubation, supernatants were harvested and stored at -80ºC for cytokine analysis until use.

Cells were harvested and washed with FACS Buffer (PBS 0.5% Foetal Bovine Serum), and surface stained with a mixture of the following labelled antibodies diluted in FACS Buffer (Table 6.3) for 45 minutes at room temperature. After surface staining, cells were washed with FACS Buffer and later fixed and permeabilized with Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Pharmingen, Breda, The Netherlands). Intranuclear staining of transcription factors (Table 6.3) was performed for 45 minutes. Afterwards, the cells were washed before their final reconstitution in PBS. Following the staining, cells were passed through a FACSVerse™ (BD Biosciences, Breda, The Netherlands) using the BD FACSsuite™ software. Fifty-thousand events per sample were acquired to perform the analysis. Unstained cells, stained samples with viability marker (BD Horizon™ Fixable Viability Stain 450 (FVS450), Breda, The Netherlands), 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 analyses according to the pattern obtained by the fixable cell-viability staining. The gating strategies can be referred in the appendix (Supplementary figure 4-6).

Table 6.3. List of antibodies for the detection of surface markers used in the study for the flow cytometry immunophenotyping.

Marker	Isotype	Clone	Conjugation	Brand	Dilution
Surface markers					
CD3ε	Mouse IgG2a	BB23-8E6-8C8	PE-Cy7	BD Pharmingen	1:200
CD4[1]	Mouse IgG2b	74-12-4	Alexa Fluor 647	BD Pharmingen	1:200
CD4[2]	Mouse IgG2b	74-12-4	PerCP Cy5.5	BD Pharmingen	1:200
CD8a	Mouse IgG2a	76-2-11	FITC	BD Pharmingen	1:150
CD21	Mouse IgG1	B-Ly4	BV421	BD Pharmingen	1:20
CD27	Mouse IgG1	B30C7	APC	BIO RAD	1:20
CD154* (CD40L)	Mouse IgG1	5c8	Mix n' stain CF405L (Biotium, Fremont, CA, USA)	BIOxCELL	1:100
CD172a (SWC3)	Mouse IgG1	74-22-15	PE	BD Pharmingen	1:200
γδTCR	Rat IgG2a	MAC320	APC	BD Pharmingen	1:200
Transcription factors					
Tbet*	Rat IgG1	4B10	BV421	SONY, BioLegend	1:100
GATA3*	Rat IgG2a	TWAJ	PE	Invitrogen	1:30
RORγT*	Rat IgG2b	AFJ9K	PerCP-eFluor 710	Invitrogen	1:160

[1] Surface staining of stimulated PBMCs [2]Surface-staining of circulating PBMCs panel. *References for non-porcine targeted antibodies: CD154 [260], RORγT [261], Tbet and GATA3 [262,263].

Cytokine secretion profiles

Harvested supernatants from *in vitro* stimulated PBMCs were used for IFNα, IFNγ, 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, Bender MedSystems GmbH, Vienna, Austria) following manufacturer's indications. Supernatants were analyzed using a Luminex® 200™ Analyzer (Luminex Corporation, Austin, TX, USA). Cytokine concentrations were determined using xPONENT® software.

Statistical analysis

All graphs and statistical analyses were performed using Prism v9 (GraphPad Software, San Diego, CA, USA). The normal distribution of data was checked using a Shapiro-Wilk test. Raw data was normalised when needed with a ln (log) transformation. Statistical differences were analysed using ANOVA. Afterwards, the means of each group were compared to the unvaccinated group using Dunnett's test. Statistical significance was represented as it follows in each graph: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical tendency was denoted as ns or • $p<0.1$.

Results

Subunit vaccines failed to protect piglets against a lethal challenge with S. suis

At the beginning of the study, five animals from different groups needed the application of HEP due to unspecific clinical signs as diarrhoea not associated with *S. suis* infection. Afterwards, the health status of the animals improved, and no clinical signs related to other pathologies were detected during the study. Only animals vaccinated with SS10/Specol® developed swelling and vascularisation in the injection sites of their hind legs (Supplementary figure 7 of the appendix), confirmed on necropsy as granulomatous-like lesions that remained throughout all the study (Supplementary figure 8 of the appendix).

Intranasal challenge with the SS10 strain induced severe clinical signs in all unvaccinated and subunit-vaccinated animals, which displayed pyrexia, apathy, anorexia, tremor, lameness, and even sudden deaths. These severe symptoms made necessary the application of euthanasia due to HEP between 2 and 6 dpi (Table 6.4). On the contrary, the bacterin SS10/Specol® vaccinated group only had one animal with abovementioned clinical signs that needed euthanasia at 3 days post-inoculation (dpi). The rest of the SS10/Specol® group stayed healthy without clinical signs until the end of the study.

Table 6.4. Compatible clinical signs of Streptococcus suis infection observed in the animals after the challenge with SS10 strain.

Group	Termination day	Clinical signs				
		Pyrexia	Anorexia	Apathy	Lameness	Neurological signs
Non-vaccinated/ Challenged (PBS NV/C)	2	+	+	+	+	-
	3	+	+	+	+	-
	6	-	-	-	-	+
	6	-	-	-	-	-
MRP2-C05	2	+	+	+	+	-
	2*	+	+	+	+	-
	2	+	+	+	+	-
	3	+	+	+	+	-
	3	+	+	+	+	-
MRP2-C05/CAF®01	2	+	+	+	+	-
	2	+	+	+	+	-
	2	+	+	+	+	-
	3	+	+	+	+	-
	3	+	+	+	+	-
	3	+	+	+	+	-
	4*	+	+	+	+	-
	5*	+	+	+	+	-
MRP2-C05/CDA	3	+	+	+	+	-
	3	+	+	+	+	-
	3	+	+	+	+	-
	3	+	+	+	+	-
	4*	+	+	+	+	-
	5*	+	+	+	+	-
	6	+	+	+	+	-
SS10/Specol®	3	+	+	+	+	-
	8	-	-	-	-	-
	8	-	-	-	-	-
	8	-	-	-	-	-
	8	-	-	-	-	-
	8	-	-	-	-	-
	8	-	-	-	-	-

*Sudden deaths

As it can be observed in Figure 6.2, mortality was higher in control and MRP2-C05 vaccinated animals. Sudden deaths were also detected in these groups with one loss on 2 dpi in the unadjuvanted antigen group, and two losses in both CAF®01 or CDA on days 4 and 5 dpi. On the contrary, SS10/Specol® vaccinated animals had less mortality in comparison with the rest of groups (p <0.001).

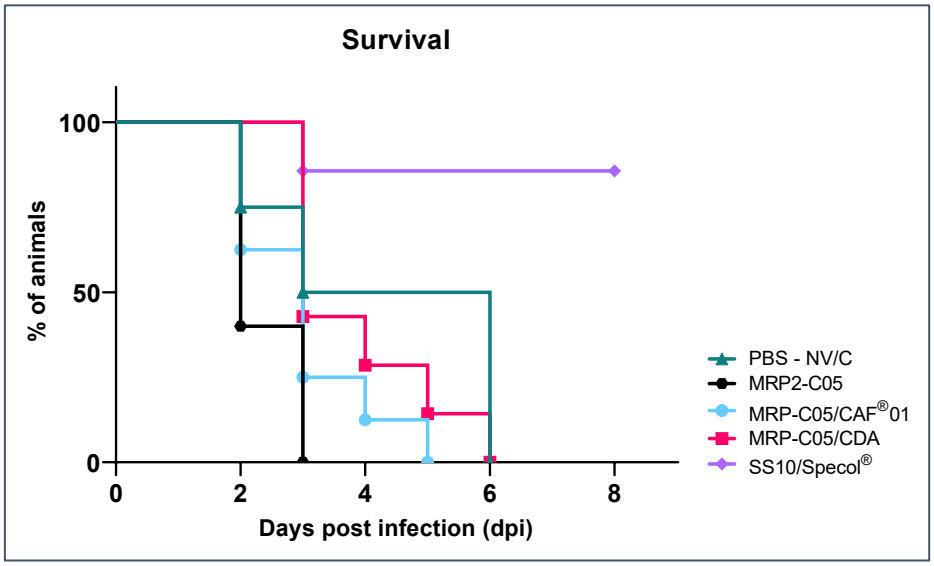


Figure 6.2. Survival curve of the experimented groups. Each line represents one vaccinated group of piglets. After the challenge, animals from the unadjuvanted antigen group died or were humanely euthanised between days 2 and 3 dpi. Piglets vaccinated with adjuvanted MRP2/C05 were mostly euthanised between days 2 to 6 dpi as it happened with unvaccinated animals. On the contrary, animals immunized with autogenous vaccine survived the experiment with only one animal euthanized on 3 dpi.

Animals vaccinated with SS10/Specol® had fewer S. suis compatible lesions and lower bacterial scores

Euthanized animals were necropsied to evaluate the extension of the lesions caused by the *S. suis* infection. Fibrinous peritonitis, pleuritis and pericarditis were generally found in piglets of all groups as well as fibrinous-purulent arthritis (Supplementary figure 9 of the appendix). However, cases of meningitis detected macroscopically and confirmed through the isolation of the challenge bacteria by culture and the following verification by MALDI-TOF were only observed in two animals from the unvaccinated and CAF®01 vaccinated groups, respectively. Although not significant, subunit-vaccinated animals euthanized from 3 dpi onwards had higher pathological scores in comparison to unvaccinated or bacterin vaccinated pigs. The animals vaccinated with SS10/Specol®, together with a higher survival rate, displayed fewer lesions than the animals from the rest of experimental groups (Figure 6.3A)

Similar outcomes of *S. suis* reisolation were detected among groups. Thus, bacterial scores in MRP2-C05 vaccinated animals were mostly variable, whereas reisolation of *S. suis* was generally reduced in bacterin immunized piglets (Figure 6.3B).

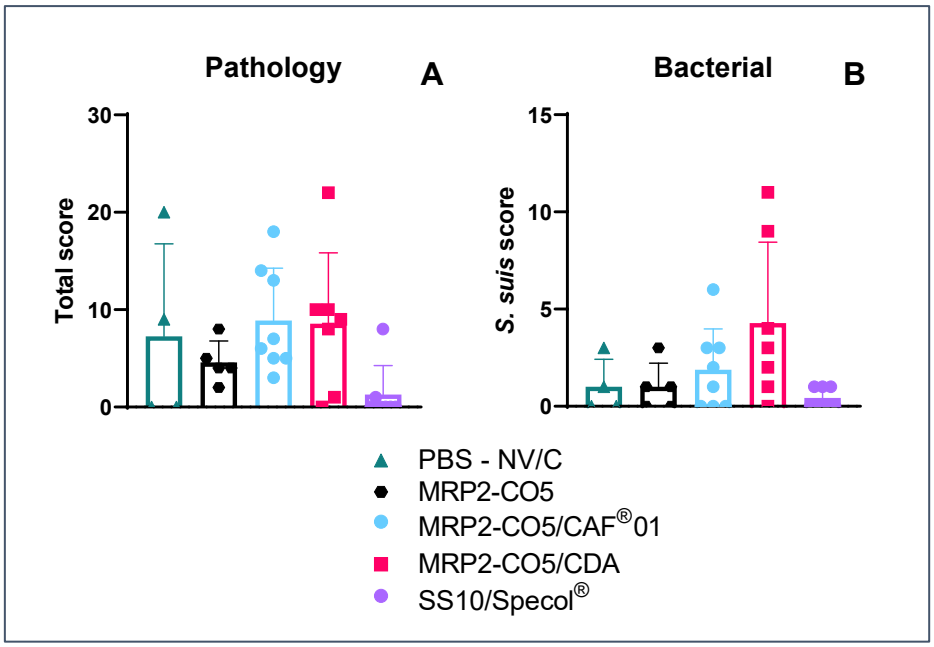


Figure 6.3. Individual gross-pathology and bacterial scores obtained in the different vaccinated groups after the lethal challenge with S. suis SS10. Averages and standard deviations are also plotted.

Piglets were colonized by serotype 9 of S. suis during the study

According to the results, animals became colonized during the study by serovar 9 of *S. suis*. This colonization was relatively homogeneous among groups except CAF®01 vaccinated animals, which showed a high dispersion. The bacterial load of serovar 9 remained invariable after challenge with serovar 2 among groups except for SS10/Specol® vaccinated animals, which slightly increased in comparison to unvaccinated piglets ($p<0.01$) (Figure 6.4A).

Serotype serovar 2 was not detected at any timepoints before the challenge. After the experimental infection, no differences in tonsil

colonization by this serotype cps2 were observed among the experimental groups (Figure 6.4B).

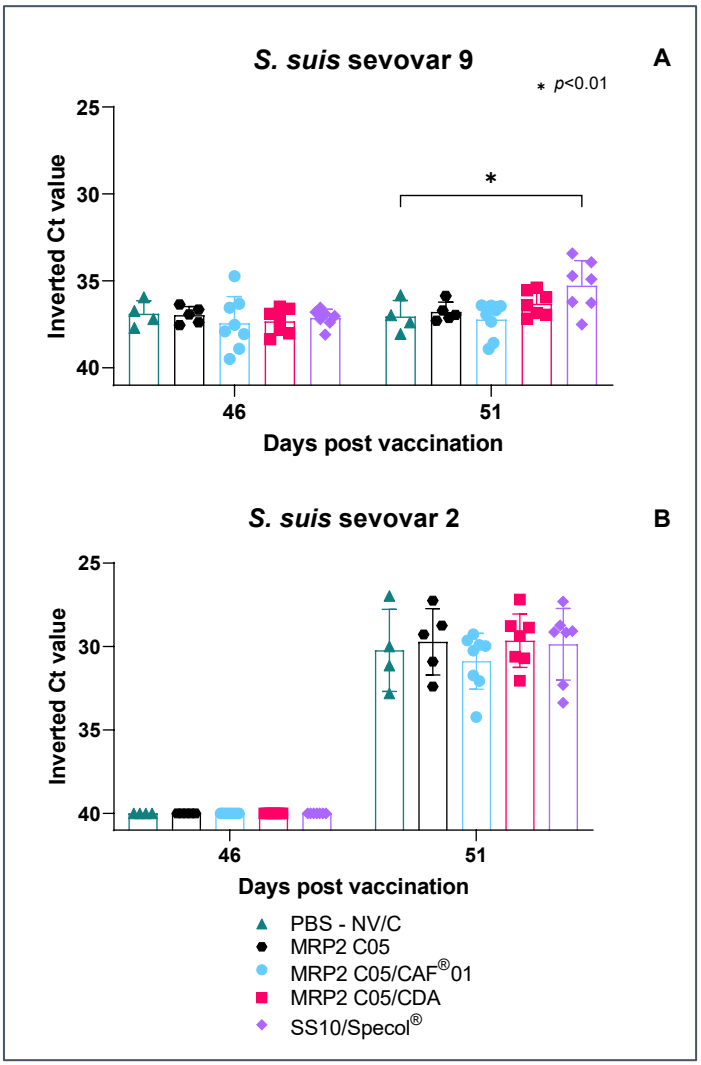


Figure 6.4. Tonsillar colonization of the experimented animals with serovars 9 and 2 of S. suis represented by Ct values obtained by serovar 9 (A) and serovar 2 (B) qPCR.

Subunit-vaccine formulations did not elicit a significative specific immune response

Antibody levels against challenge strain SS10 showed different results between groups. Only animals vaccinated with the bacterin SS10/Specol® elicited a significant anamnestic response after the second immunization in comparison with the unvaccinated animals ($p<0.001$). On the contrary, antibody levels against whole bacteria remained in the basal level in subunit-vaccinated and unvaccinated groups (Figure 6.5A).

Variable responses were detected in specific antibodies against subunit antigen MRP2. Again, vaccinated animals with SS10/Specol® showed higher antibody levels when compared to the unvaccinated group ($p<0.001$). Subunit-vaccinated animals with CAF®01 or CDA adjuvants developed weak responses after booster with slight increases in some animals but without significant differences. These transient responses disappeared after the challenge when they dropped to the basal level (Figure 6.5B).

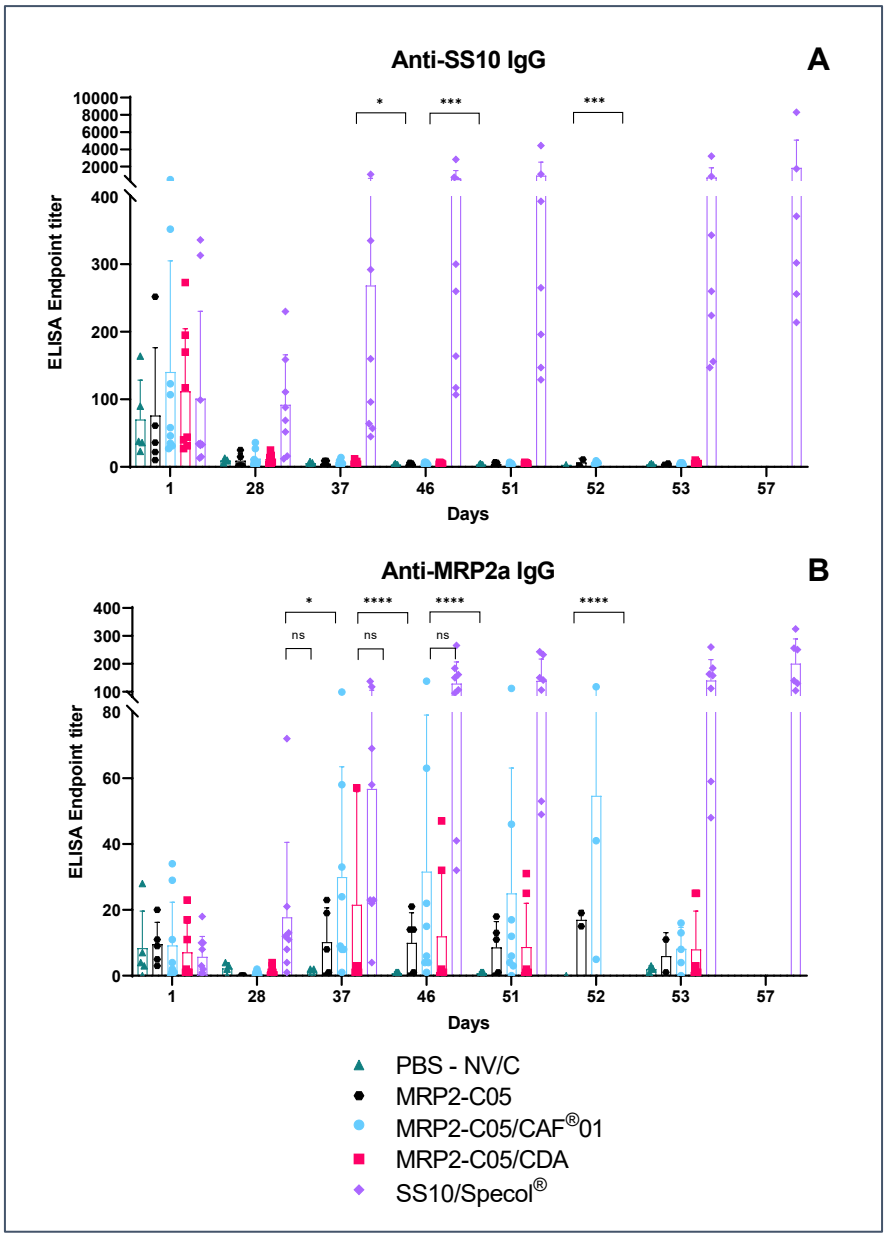


*Figure 6.5. Antibody response against inactivated S. suis strain SS10 (A) and subunit protein MRP2 (B) of the different sampled timepoints. Averages from ELISA endpoint titres of each experimental group are represented in bars together with standard deviations. Single dots represent the individual values obtained from animals of each group were performed using Dunnett's test. Statistical significance was denoted as it follows in each graph: ns p<0.1, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

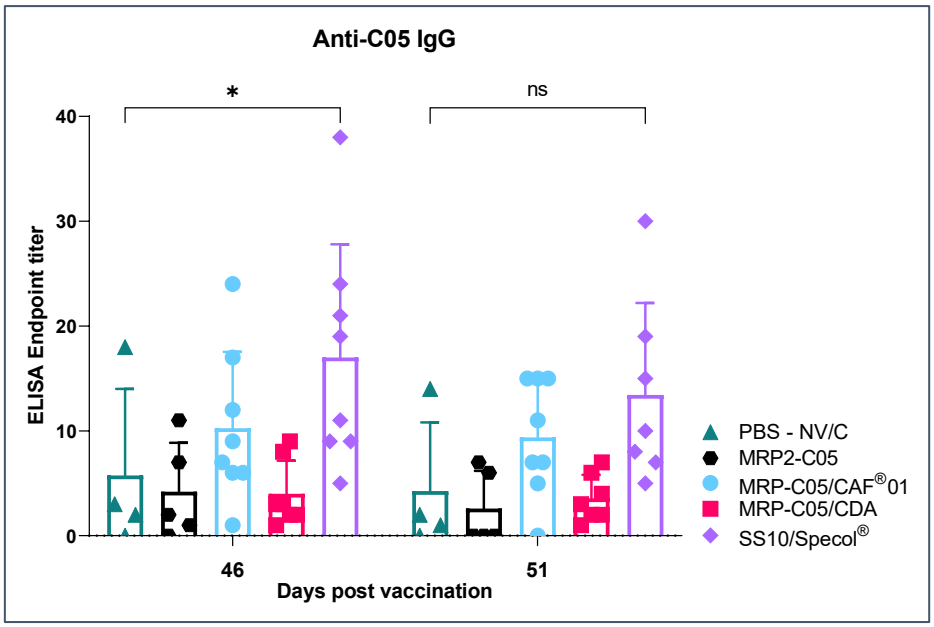


*Figure 6.6. Antibody response against C05 antigen measured from sera samples collected before (D46) and after challenge (D51). Averages from ELISA endpoint titres of each experimental group are represented in bars together with standard deviations. Single dots represent the individual values obtained from animals of each group. were performed using Dunnett's test. Statistical significance was denoted as it follows in each graph: ns p<0.1, * p < 0.05.*

Specific anti-C05 response was evaluated in two timepoints, on days 46 and 51, before and after challenge. Variable responses were detected among the experimental groups. Only the piglets vaccinated with SS10/Specol® elicited higher response before the challenge (p=0.03). On the other side, animals vaccinated with subunit combinations had variable antibody values, with the pigs vaccinated with CAF®01 showing higher titres although without significance in comparison to the unvaccinated group. After challenge, titres of antibodies slightly decreased in all the experimental groups but maintaining the

tendencies; the significance observed on day 46 with the SS10/Specol® group turned non-significant by day 51 (Figure 6.6).

Opsonophagocytosis (OPKA) assay

Small variations in bacterial survival after incubation with sera and neutrophils without statistical significance were observed among groups. Piglets vaccinated with SS10/Specol® showed a tendency to increase the killing capacity with the neutrophils of animal 1 but not with animal 2 (Figure 6.7).

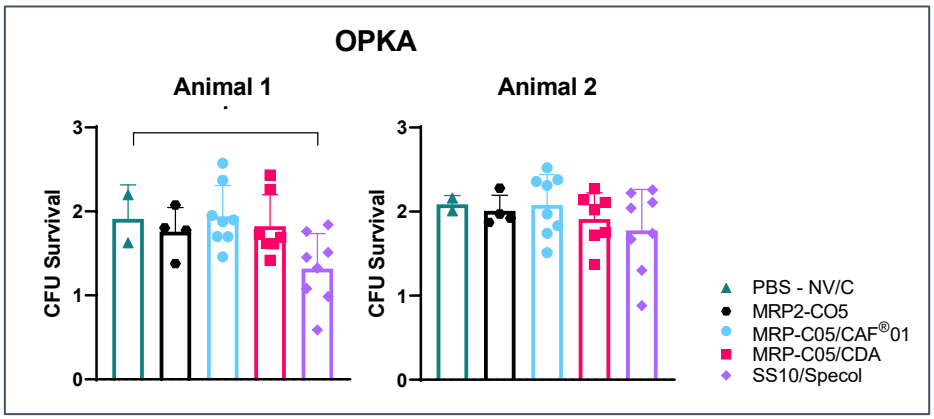


Figure 6.7. Opsonophagocytosis and killing assay performed with porcine neutrophils collected from healthy animals and an external source of complement. Individual values are represented in dots, averages and standard deviations are also plotted. Statistical tendency was denoted as • p<0.1.

Cell-mediated immune response

Differences between groups were detected in some white cell blood populations one day after the first immunization. Animals vaccinated with SS10/Specol®, had less percentages of circulating B, T cells and monocytes ($p<0.05$), but a high percentage of granulocytes ($p<0.0001$) in comparison with subunit-vaccinated and unvaccinated groups. No differences between vaccinated or non-vaccinated groups were observed in the remaining timepoints, and trends during the experiment remained similar among groups. A decrease in granulocytes and Th helper cells was observed in all the groups. On the other hand, increasing tendency was observed in memory T cells and CTL cells until the boost followed by a decrease. However, none of these differences were found significant between groups (Figure 6.8).

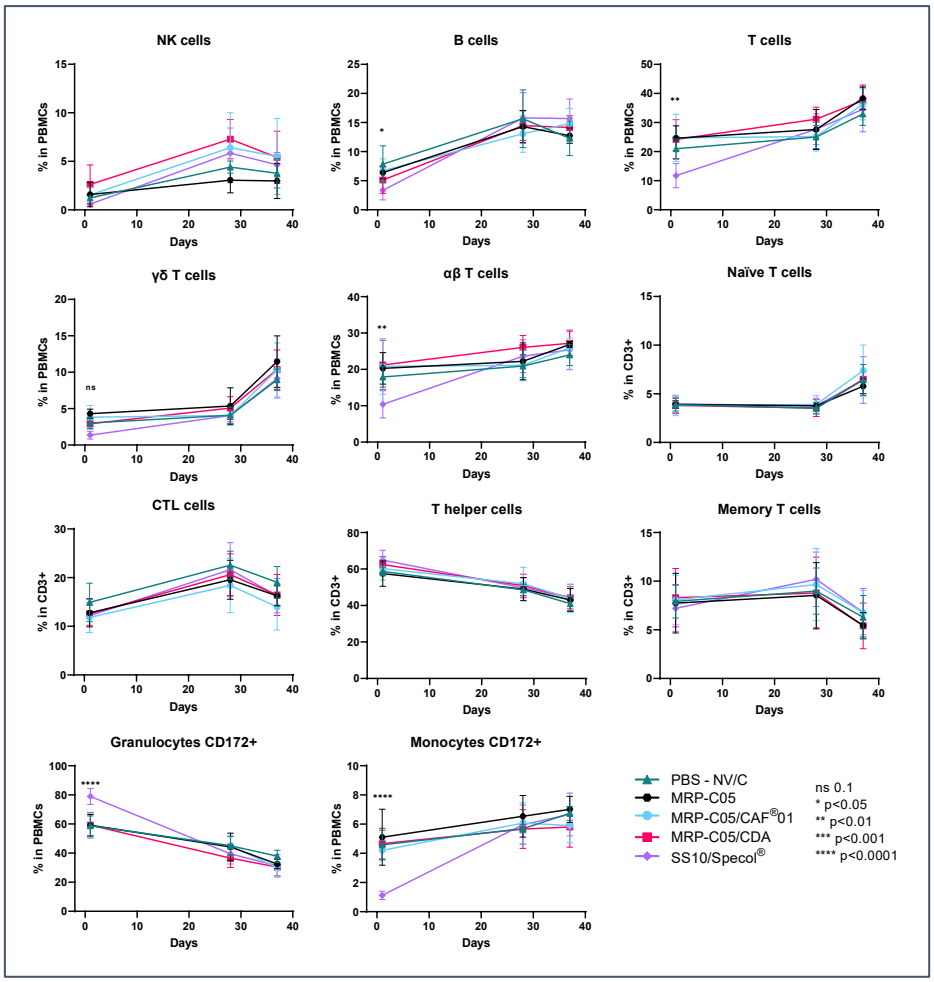


*Figure 6.8. Main circulating white blood cell populations from experimental animals were measured by flow cytometry before the challenge with S. suis SS10 on days 1, 28 and 39 post-vaccination. Statistical significances were denoted as it follows in each graph: ns p<0.1, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Memory and specific immune responses, evaluated by the *in vitro* restimulation of isolated PBMCs with vaccine subunits and formalin inactivated SS10 strain, showed variable outcomes among groups, hindering the obtention of evident effects. Even though, some non-statistical differences between groups could be glimpsed. Populations

of T helper cells (CD3+CD4+) remained in a similar variable level upon the stimulation of both MRP2 and Bacterin. Some increase in variability can be observed in vaccinated animals.

Th1 cells measured through the expression of Tbet transcription factor were highly diverse in all vaccinated and unvaccinated groups after stimulation of MRP2 but not with bacterin, where SS10/Specol® animals have a slight increase.

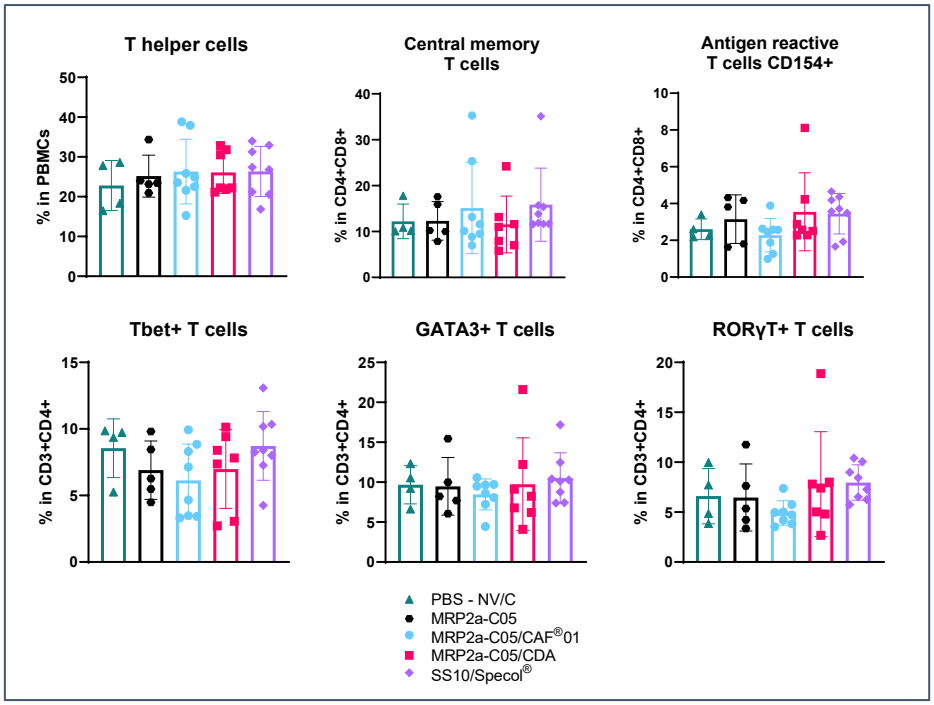


Figure 6.9. T cell subsets evaluated by flow cytometry after 48-hour in vitro stimulation of PBMCs isolated at 46DPV with MRP2 protein.

Th17 cells, expressing RORγT as the master regulator, showed an increasing tendency with bacterin stimulation ($p>0.1$) in animals

vaccinated with SS10/Specol®; and increased values without significance were observed in animals vaccinated with CDA and Specol® upon the stimulation with MRP2. Some animals from vaccinated groups had high percentages of central and antigen reactive memory T cells, without again significance with both stimuli (Figure 6.9 and Figure 6.10). Th2 cells measured by the expression of GATA3 unlike Th1/Th17 subsets, remained mostly invariable in all groups upon both stimulations.

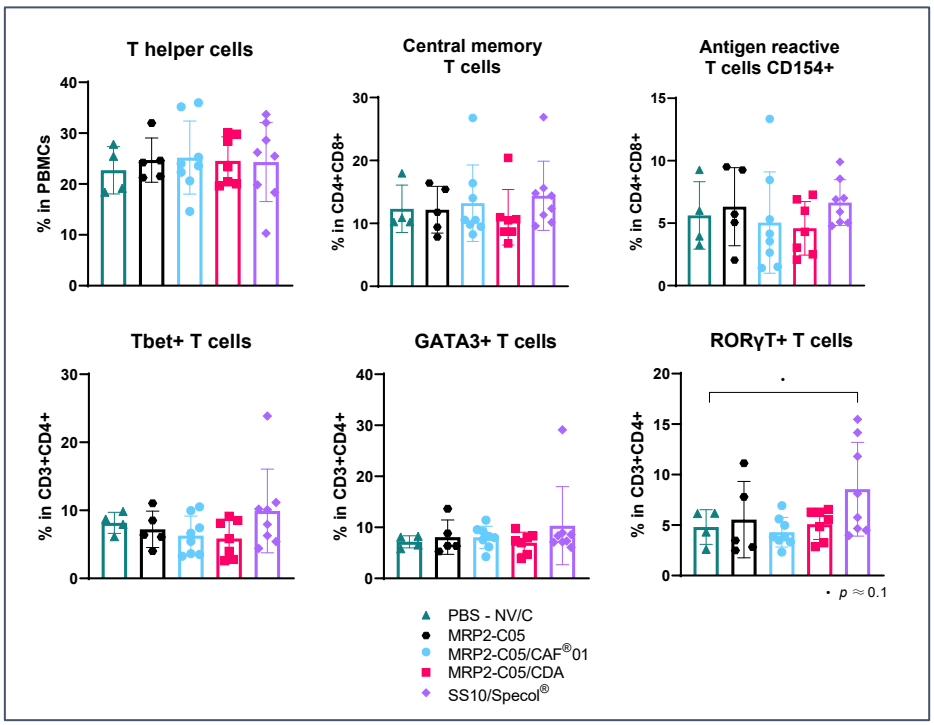


Figure 6.10. T cell subsets evaluated by flow cytometry after 48-hour in vitro stimulation of PBMCs isolated at 46DPV with formalin-inactivated strain SS10. Statistical tendency denoted as follows: • p value < 0.1.

Cytokine profiles of in vitro stimulated PBMCs

Analysis of the supernatants collected after the in vitro stimulation of PBMCs with subunit protein MRP2 or formalin-inactivated strain SS10 of S. suis revealed a relatively low and variable cytokine profiles. After the stimulation with MRP2, only the PBMCs of few piglets from the SS10/Specol® group secreted remarkable amounts of IFN-α, IFN-γ, IL-12/IL-23p40 and TNF-α. The rest of vaccinated or unvaccinated groups did not generate significant changes for these cytokines. Variable findings were observed in the rest of the cytokines with low profiles for IL-1β, IL-10 and IL-6 for CAF®01 combination. In addition, high secretion of IL-4 was observed in animals vaccinated with SS10/Specol® (Figure 6.11).

These tendencies observed in MRP2 stimulated PBMCs seemed to be increased when the cells were stimulated with the whole inactivated bacteria. Piglets vaccinated with CAF®01 combination showed downregulation in IL-6 ($p=0.005$) and IL-10 ($p=0.01$). This tendency of downregulated cytokines in the CAF®01 group in comparison to the rest of the groups was also observed, but without statistical significance, in IL-1β, IL-4, IL-6, IL-10, IL-12/IL-23p40 and TNF-α; except for IFN-α and IL-8 (Figure 6.12).

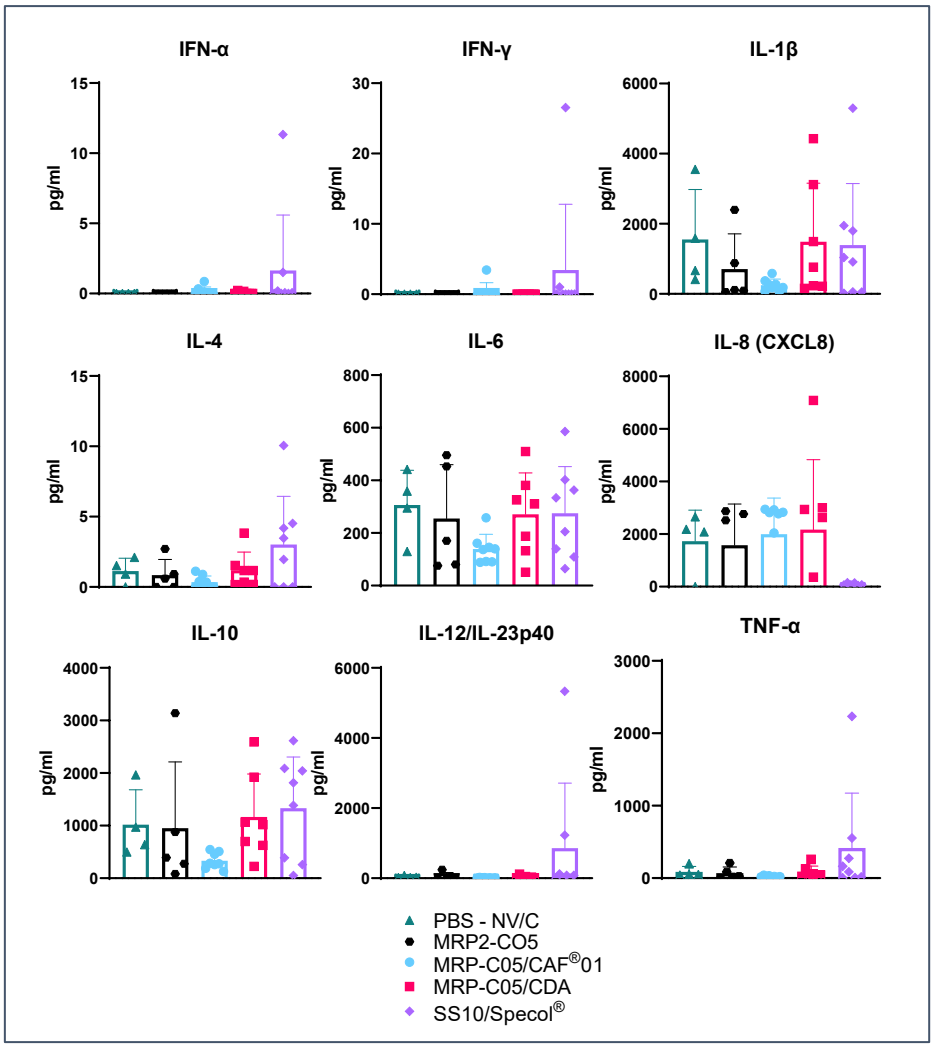


Figure 6.11. Cytokine profile obtained by ProcartaPlex™ immunoassay from the supernatants of 46DPV PBMCs stimulated with MRP2 protein.

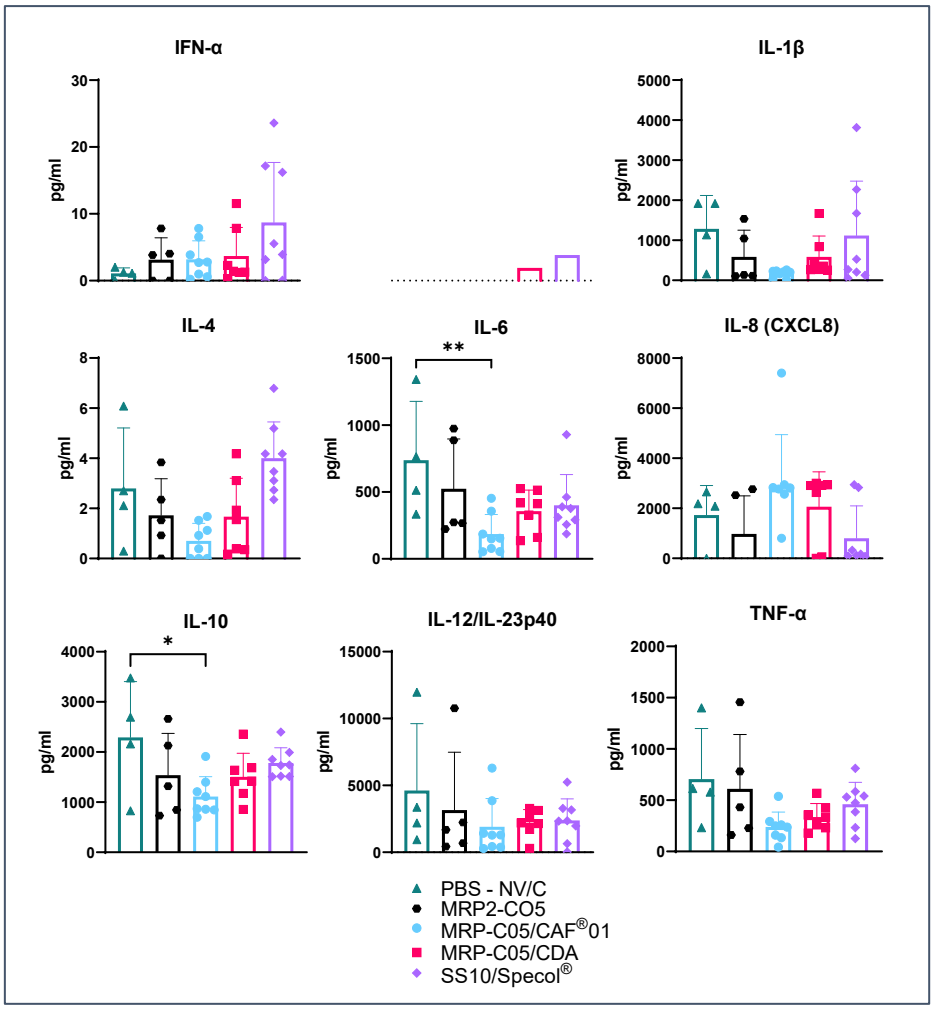


*Figure 6.12. Cytokine profile obtained by ProcartaPlex™ immunoassay from the supernatants of 46DPV PBMCs stimulated with formalin-inactivated S. suis SS10 strain. Statistical significances are denoted as it follows in the corresponding graph * $p < 0.05$, ** $p < 0.01$.*

White blood cell counts during the infection process

White blood cells (WBC) were measured with a hemacytometer to evaluate possible leucocytosis or leukopenia in the animals during the infection. A general increasing tendency on WBC counts was observed in all the groups except in the animals vaccinated with SS10/Specol®, which moderately increased on the first three days and became stabilized and decreased in the final days of the experiment (Figure 6.13).

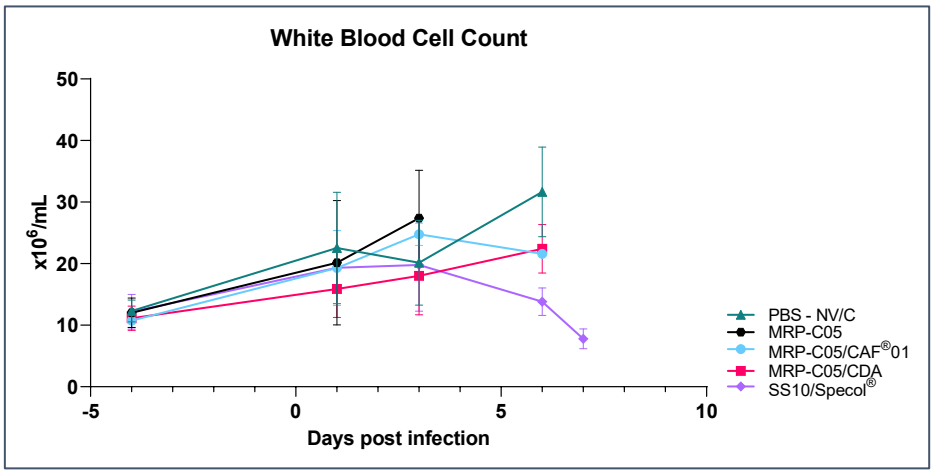


Figure 6.13. WBC counts during the experimental infection. Samples were obtained from animals on days -4, 1, 3, 6 and 7 after the infection with S. suis serovar 2. Mean of values are represented in dots with standard deviation in lines.

Discussion

Neonate vaccination is the most effective tool to combat early life infectious pathogens. Even though, it seems that some diseases are difficult to be controlled by vaccines, and swine streptococcal disease is one of the most notorious examples. The requirement to reduce the use of antimicrobials and the inexistence of available licensed *S. suis* vaccines in Europe, force the practitioners to use autogenous vaccines. Unfortunately, these preparations are not tested for safety or even immunogenicity, leading to limitations and uncertainties for its use for a proper control of the disease. The complexity in the pathogenesis of *S. suis* infection, where multiple virulence factors are involved and the disease is considered multifactorial, makes the finding of an effective subunit vaccine a challenging quest. Since autogenous vaccines normally offer homologous and effective immune responses, it is established that the generation of opsonizing antibodies represents a correlate of protection in *S. suis* infection [264]. One of the current approaches for the search of effective and cross-protective subunit vaccines is based on the immunization combining different *S. suis* proteins to cover several antigens that allow an efficient opsonization of the bacteria. Several subunit vaccine candidates against *S. suis* have been tested so far, although most of them are still in exploratory or preclinical stages [86]. For this reason, in this study we aimed to evaluate two new formulated antigens from the *S. suis* surface, the MRP2 and C05 antigens, adjuvanted with two novel adjuvants, CAF®01 and CDA. In addition, to better determine and understand the immunogenicity and efficacy after a lethal challenge with virulent *S.*

suis, an autogenous vaccine with formalin-fixed *S. suis* serovar 2 emulsified with Specol® immunogenic adjuvant was also included.

After the immunization with the CAF®01, CDA or autogenous experimental vaccines, different cell-mediated and humoral immune responses were recorded. However, MRP2-C05 adjuvanted formulations with CAF®01 or CDA failed in providing robust responses and thus protection to the vaccinated animals after the challenge. Generalized cases of fibrinopurulent polyserositis and arthritis were observed in most of the animals immunized with subunit antigens, indicating a weak immune response uncapable to tackle the systemic infection produced by *S. suis*. The autogenous vaccine (SS10/Specol® combination), on the contrary, was the only vaccine of the study which protected most of the animals and elicited a significative immune response, including antibodies against both subunit proteins MRP2 and C05.

Reviewing the literature, some explanations can help to elucidate the results obtained in the present study regarding the limited immune responses we obtained with both subunit antigens MRP2 and C05, even when in previous experiments these antigens elicited protection and immunogenicity together with other immunogens. This is the case of a vaccination study in pigs immunized with different combinations of adjuvanted MRP and extracellular factor (EF), another virulence-related protein of *S. suis* [112]. Immunization with MRP alone resulted in the generation of an antibody response that did not provide significant protection in comparison with those animals vaccinated with MRP

combined with EF. Yet, this fact indicated a priori that the vaccination of a sole antigen of *S. suis* might not provide protection against a challenge, suggesting the use of antigen combinations to get protective responses. In addition, the authors also suggested the important function of adjuvants in triggering an effective response, since the piglets vaccinated with the mixture of MRP+EF adjuvanted with Specol® emulsion elicited better immune responses than the ones vaccinated with the same mixture but combined with Alum [112].

On the other hand, the C05 antigen named SSU0185 tested together with other five immunogens in mice and pigs and formulated with adjuvants, provided significant humoral and cell-mediated responses, and offered protection against lethal challenge with *S. suis*. In the pig experiment [114], the animals that developed the best immune response were vaccinated with the oil-in-water Emulsigen® adjuvant, which generates a tissular depot with droplets of about 2 μm that favour its processing and subsequent antigen presentation by dendritic cells. In the study conducted in mice [115], animals immunized with the antigen mixture adsorbed onto biopolymer particles and formulated with Quil-A® adjuvant, demonstrated increased specific-antibody titres and protection against lethal challenge. The authors related this response to an efficient uptake of the particles (of about 300 nm of diameter approx.) by the antigen-presenting cells (APCs) along with the effect of the saponin included in Quil-A® adjuvant.

Considering the size of the antigens, and the results obtained in the mentioned studies with MRP or C05 in which they used emulsion or

particulate adjuvants, the retention of the immunogens in the tissue after the injection might be critical to develop an efficient immune response to allow their capture by the APCs. According to our results, this antigen retention and APC uptake was not achieved with the CDA formulation or at least was partially accomplished in the case of CAF®01. In fact, 6 out of 8 piglets vaccinated with CAF®01 adjuvanted antigens elicited a transient and non-homogeneous antibody response against both antigens, whereas one or two animals of the CDA group reacted in anamnestic way to the immunogens. These contrasting responses can be explained by the mechanism of action of both adjuvants, since CAF®01 forms a transient depot in the injection site, while CDA is administered in a soluble form into the tissue. Theoretically, the size of our antigens MRP2 and C05 might not represent a limitation for its combination with both novel adjuvants. However, the physicochemical composition of the antigens is critical for their adsorption into particulate adjuvants and therefore the generation of efficient immune responses [265]. This fact become crucial when the antigens are formulated onto the liposomal surface of CAF®01 [224,266]. We ignore the behaviour of the combined *S. suis* antigens within CAF®01 and we cannot discard a formulation impairment due to their physical properties that impedes a stable liposomal structure and therefore a correct intake by the APCs. In parallel to these issues concerning the antigen/liposome formulation, it cannot be ruled out that the immune response triggered by the sole activation of Mincle through TDB contained in CAF®01 would be sufficient to cope the infection. In this respect, this suboptimal Mincle receptor activation

might be enhanced with the addition of TLR2 ligands that tailor the immunity to an adequate response against gram-positive bacteria as reported previously [267,268].

Regarding the CDA adjuvant, the intramuscular administration performed in the present experiment proved to be less efficient to obtain a relevant immunogenic profile than mucosal route as confirmed for cyclic dinucleotides in other studies [223,269]. To our opinion, encapsulation of CDA and other STING ligands in a delivery system such as liposomes or adsorbed onto particles might become a future strategy for its administration by parenteral routes to obtain stability and better immunogenic responses, as reported previously in pigs [215] and mice [270].

In this study, upregulation of proinflammatory cytokines upon the *in vitro* stimulation with formalin-inactivated serotype 2 of *S. suis* was observed in most of the vaccinated groups in agreement to previously reported studies [271,272], except for CAF®01 vaccinated animals. Comparing to the rest of the groups, CAF®01 vaccinated piglets showed a general trend of downregulation in most of the cytokines and significatively for IL-6 and IL-10. It is known that *S. suis* stimulates the secretion of TNF-α, which subsequently induces the upregulation of proinflammatory cytokines such as IL-6 and IL-10 [273]. In contrast, it has been reported that decreased levels of IL-6 in blood protects pigs against pneumococcal septicaemia [274]. To our knowledge, the cytokine decrease observed after the *in vitro* stimulation with either subunit MRP2 or whole inactivated bacteria, together with the variable

outcomes regarding specific subunit protein antibodies and variable levels of colonization, may indicate at least a partial immunization. Although this limited immunization did not protect against a challenge with serovar 2 SS10 strain, it would be important to explore this effect of CAF®01 to avoid the harmful effects of the cytokine storm occurred in a systemic infection with *S. suis*.

SS10/Specol® vaccine, conversely to the subunit formulations, elicited an efficient immune response with increased antibodies and detectable cell-mediated immune responses. The generated immunity was capable to protect the vaccinated animals with less clinical signs and gross lesions. In fact, only two animals immunized with this autogenous vaccine manifested clinical signs and succumbed after the lethal challenge with serovar 2 of *S. suis*. According to the results obtained in the circulating cell subsets measured by flow cytometry, the cell recruitment ability of Specol® seemed unrivalled in comparison to the two subunit vaccines used in this study. In addition, the antibody repertoire generated after the vaccination with the adjuvanted bacterin was much higher than the elicited by subunit vaccines. Indeed, significative antibody titres against both MRP and C05 were obtained in bacterin vaccinated animals, this fact confirms the presence of these antigens on the pathogen surface. Interestingly, the protection afforded by Specol® combination did not correlate with opsonophagocytosis despite having a high level of antibodies. This fact could be explained because the test was performed mainly with granulocytes, excluding the effect of macrophages and other phagocytic cells in the assay. However, not all the qualities observed in Specol® combination seemed

to be appropriate for a vaccine formulation. Likewise, the granulomatous lesions generated after vaccination lasted until the end of the study, making its use undesirable for animals due to animal welfare reasons. These kind of lesions were previously reported in other vaccination studies with different species after intramuscular injection [275–277]. In addition, and as characteristic of all autogenous vaccines independently for the adjuvant contained, the protection achieved through the vaccination by a given serovar would not cross-protect against other virulent serovars [278].

Sometimes experimental antigens from *S. suis* do not provide neither sufficient protection nor strong immune responses [279,280], and this would be the case in the present study. Although the tested antigen/adjuvant formulations did not elicit effective or measurable immune responses, our results could pave the way to study more thoroughly the antigenicity of both MRP2 and C05. An exhaustive analysis of each antigen will be further needed to take conclusive results and dissect the type of adjuvant that suits better to their properties in order to achieve protective responses in neonate pigs.

In summary, the lethal challenge of *S. suis* affected all the groups at different degrees since compatible clinical signs were generally observed in all the experimented groups and were lately confirmed at post-mortem examination. This clinical signs, pathology and lower survival correlated with the weak humoral responses observed in those MRP2 and C05 vaccinated groups indicating an unprotective immunity. In addition, the cell-mediated immune responses measured in

circulating PBMCs from the subunit-vaccinated piglets were in general basal apart from CAF®01. Indeed, down-regulation of some proinflammatory cytokines in CAF®01 vaccinated animals deserve extended studies of cell-mediated immunity not only at systemic but also at mucosal and lymph node levels to better dissect the effect of the adjuvant together with both antigens. This knowledge will help to understand the immune responses underlying protection for *S. suis* disease.

Conclusions

Piglets vaccinated with MRP2-C05 antigens adjuvanted with CAF®01 or CDA, failed to elicit robust immune responses capable to cope against a challenge with serovar 2 of *S. suis*. Specol® adjuvanted autogenous vaccine delivered a strong antibody response correlating with protection. Further studies of antigen evaluation are needed to choose a suitable adjuvant and therefore obtain efficient and immunogenic vaccine combinations.

Chapter VII. General discussion

Chapter 7. General discussion

Adjuvants are considered indispensable compounds for the elaboration of immunogenic and effective vaccines, especially with subunit antigens, which often lack the necessary immunogenicity to trigger an efficient response. Nonetheless, the nature of the antigen is an important factor to consider when choosing an adequate adjuvant to formulate effective and immunogenic vaccines. In this PhD thesis, three animal experiments were carried out for the evaluation of the immune responses triggered by conserved antigens from three different pathogens. All the experiments shared the animal model (in this case the pig) and the adjuvants included in the vaccine formulation were the liposome-based CAF®01 or the cyclic dinucleotide CDA. CAF®01 and CDA are well-characterized adjuvants tested previously in different animal models, which can be used either by parenteral or mucosal routes retaining their immunostimulatory effect [150,197,198,281]. In the studies presented in this PhD Thesis, different experimental vaccines were intramuscularly injected in the swine models assayed.

Table 7.1. Summary of results obtained with the use of CAF®01 and CDA adjuvants in the three studies of this PhD dissertation.

Responses	Studies and adjuvant formulations					
	Influenza pdmH1N1 (Chapter III)		*G. parasuis* (Chapters IV and V)		*S. suis* (Chapter VI)	
	CAF®01	CDA/ αGCM	CAF®01	CDA	CAF®01	CDA
Pathogen-compatible lesion reduction	++	-	-	-	-	-
Pathogen load reduction	+++[1]	-[1]	++[2]	-[2]	-[3]	-[3]
Specific humoral response (IgG)	+++	-	-	++	+	-
Humoral mucosal response (IgA)	-	+	-	-	ND	ND
Cell-mediated immune response	++	+	-	+	+	-

[1] Nasal and lung load. [2] Nasal colonization. [3] Tonsil burden. ND: not evaluated.

In general, CAF®01 adjuvant elicited the characteristic Th1/17-type immune responses described in previous studies [131,282]; this response is attributed to the immunostimulatory effect of the TDB through Mincle interaction [131]. A significant or variable reduction in the pathogen burden of the animals vaccinated with CAF®01 combinations was observed in the lung or nasal cavities in animals immunized with NG34 and F4 antigens (Table 7.1). This decrease in pathogen load suggests mucosal priming as previously reported [136,197,225]. To our opinion, the induced protective and mucosal immune responses observed in the vaccines formulated with CAF®01

needs further attention, since enhanced mucosal priming may help in the prevention of opportunistic diseases such as the ones studied in the present Thesis.

Nonetheless, the immune responses following vaccination using CAF®01 adjuvant can still be improved. In this respect, a liposome stability assessment of the generated particles during vaccine preparation would be key for the generation of effective immune responses as mentioned previously. In addition, the possibility to include additional immunostimulatory molecules into the liposome formulation that may further stimulate innate immune receptors (as for example TLR) would be necessary to tailor the immune response towards a Th2-type response with stronger antibody production, if needed. The sole stimulation of the Mincle receptor with TDB seems to be insufficient to clear pathogens that possess multifactorial virulence mechanisms, such as *S. suis*.

Under our experimental conditions, CDA adjuvant administered by intramuscular injection in soluble form induced variable immune responses depending on the antigen used (Table 7.1). Thus, in Chapter III, CDA was combined with the CD1 agonist αGCM in a soluble form aiming to enhance its immunogenicity through the stimulation of invariant NK T-cells. Nonetheless, instead of the expected enhancement, variable and transient immune responses were achieved. In the case of Glässer's disease, a significant humoral immune response was elicited in the piglets immunized with the F4 protein, but the increase of specific antibody secretion was not generalized in all the

piglets of the group. In the study with *S. suis* antigens, no significant humoral or cell-mediated immune responses were detected. In agreement with previous experiments with CDA adjuvanted antigens, intramuscular injection of soluble CDA may not be the most efficient route of administration. Indeed, vaccines can display differential immune profiles according to the administration route [283]. In the case of cyclic dinucleotides, Landi and colleagues referred to superior immune responses of soluble CDA when alternating intramuscular and intranasal administrations in accordance with unpublished observations [284]. Although most vaccines are administered by the intramuscular route, skeletal muscle tissue has a low density of immune cells in comparison to mucosae or dermis [285]. Moreover, rapid diffusion of the vaccine to the draining lymph nodes is required to obtain quick effective responses. In this respect, cyclic dinucleotides carry negative charges that prevent their diffusion across the plasma membrane to activate STING into the cytosol [286]. For this reason, in multiple experiments, cyclic dinucleotides were encapsulated or adsorbed into particles to obtain stability and efficient responses [287,288], facilitating diffusion through cell membranes. These approaches may enhance the correct uptake by dendritic cells and constitute a delivery system to apply for further vaccine formulations.

However, TDB contained in CAF®01, and STING ligands such as cyclic dinucleotides do not seem to be incompatible. The possibility to combine both immunostimulatory molecules has been explored recently in mice, cattle and pigs after immunization with inactivated foot-and-mouth disease virus vaccine [289]. Interestingly, the

satisfactory results obtained especially in pigs, reporting an enhanced long-lasting humoral response as well as neutralizing antibodies, provide possibilities to explore this combination of adjuvants with further antigens against porcine diseases.

Table 7.2. Main characteristics of the antigens used in the studies of this PhD dissertation.

Pathogen	Antigen	Protein of origin	Length (amino acids)	Weight (KDa)
Influenza virus pdm09 H1N1	NG34	Hemagglutinin H1	34	4
Glaesserella parasuis	F4 protein	Virulent trimeric-autotransporters (VtaA)	134	13.74
Streptococcus suis	MRP2	Muramidase released protein (MRP)	754	81.28
	C05 antigen (SSU0185)	Putative tagatose-6-phosphate aldose/ketose isomerase (AgaS)	389	42.61

The antigens used in this PhD work encompass diverse sizes and sources, but all of them correspond to proteins that are conserved in different strains of the corresponding pathogen and exposed in their surface (Table 7.2). As the size of the antigens increase, the complexity of their immunogenic characterization increases too. Thus, B and T cell epitopes have been identified in NG34 peptide (non-published results) and F4 protein, although partially in this last case [107]. On the contrary, the epitopes of both MRP2 and C05 are still to be determined. Previous

knowledge generated *in silico* and *in vitro* regarding immunogenicity studies is important to understand the behaviour of a given antigen when formulating vaccines. With this information, immunity can be tailored with adjuvants towards an effective response depending to the targeted pathogen.

Short antigens, in general need to be modified to enhance their immunogenicity properties. This was the case of the NG34 peptide of influenza virus, whose sequence was adjusted to ameliorate the generation of protective antibodies [97]. Despite such theoretical improvement, no HI titres prior to the challenge with the virus were generated, although protection was observed in the animals vaccinated with NG34+CAF®01. In contrast, HI titres detected seven days after the experimental infection in this vaccinated group were higher to those from the control group. According to these results, it seems that subsequent infection after NG34 peptide vaccination provides an antigen exposure that amplifies the generation of further neutralizing antibodies. To our knowledge, this effect has only been observed with animals vaccinated with NG34 and other HA-derived peptides contained in plasmid constructs [93,95]. This characteristic of NG34 peptide together with the elicited specific antibody response and increase of IFN-γ secreting cells, makes NG34 a suitable candidate to consider for future influenza virus subunit vaccines either in peptide form or in plasmid constructs.

In contrast to the NG34 peptide, the antigens used for vaccination against Glässer's and streptococcal diseases were considerably larger.

The use of proteins for immunization can help in the generation of a varied antibody repertoire but, on the contrary, can difficult the formulation of effective vaccines depending to the adjuvant used as exposed above. Thus, the F4 protein triggered an enhanced antibody response in combination with carbomer adjuvant when vaccinating sows [108]. On the other hand, *S. suis* antigens elicited differential responses when formulating MRP2 together with an emulsion adjuvant (unpublished results) or C05 antigen combined with saponins [114,115]. These facts confirms that the selection of a suitable adjuvant is key to generate an adequate and effective immune response for each disease. Nonetheless, the full potential of the antigens described in this PhD Thesis is still to be explored.

As explained in the introduction, there is a need of new strategies in the livestock industry to tackle infectious diseases, especially in those affecting younger pigs. In this context, combination of antigens to generate multivalent vaccines against post-weaning diseases like those existing for human newborns, as for instance Measles-Mumps-Rubella (MMR) or diphtheria-tetanus-pertussis (DTP), would be an excellent strategy yet to be developed. Interestingly, another approach may target the passive immunity through sow vaccination. Indeed, maternal vaccination may address the issue of the immune window susceptibility of young piglets by the generation of long-lasting antibodies capable to overlay this period and help animals counteracting infections after weaning. However, this strategy opens another "pandora box", which is the potential interference effect of maternally derived immunity when vaccinating pigs in the postweaning area [290].

Subunit vaccines are cost-effective and safe products and may represent an excellent strategy to combat infectious diseases. However, their development is often long, and several trials are necessary in the exploratory and preclinical stages before its scalation to the clinical phases. The work presented in this dissertation provides useful information to improve vaccine candidates. Even though effective subunit vaccines against the presented porcine diseases are yet to be conceived, the work described in this PhD Thesis contributes with new knowledge about them and pave the way to continue the search and refinement of new vaccine prototypes. The obtained information should help improving not only to the immunogenicity and efficacy of new vaccine prototypes, but also in valuable models for the determination of protection correlates in neonate and young piglets.

Chapter VIII. Conclusions

Chapter 8. Conclusions

1. Vaccination of pigs against influenza virus using NG34+CAF®01 elicited a strong immune response with specific antibodies against NG34 and IFN-γ secreting cells that correlated with reduction of influenza virus load in lung and decreased flu-like compatible lesions, in the absence of HI titres prior to the challenge. These features suggests that this combination provided protection against homologous pdmH1N1 IV.

2. The combination of the NG34 influenza virus peptide and adjuvants CDA/αGCM elicited a weak immune response without reduction of viral load in the lung or nasal cavities and variable pathological expression of the vaccinated animals, indicating a failure in providing protection.

3. Vaccination against *G. parasuis* using the F4+CAF®01 combination was able to clear the nasal colonization of a heterologous strain of *G. parasuis* in the absence of antibodies in sera, indicating a primed mucosal cell-mediated response.

4. Vaccination against *G. parasuis* using the F4+CDA combination elicited a variable systemic response with specific anti-F4 IgGs against *G. parasuis*, overcoming the limitations of neonate vaccination in generating antibody responses.

5. The intraperitoneal challenge with serovar 5 of virulent *G. parasuis* induced severe lesions in all experimental groups, hampering the obtention of concluding results regarding vaccine efficacy. This type of inoculation may not represent an optimal route for vaccine efficacy assessment.

6. *In vitro* stimulation of PBMCs with heat-inactivated *S. suis* modulated the expression of proinflammatory cytokines in animals immunized with CAF®01 adjuvanted MRP2/C05

animals, which also showed a weak antibody response, indicating a partial immunization. On the contrary, the combination of CDA adjuvanted MRP2/C05 did not elicit significant humoral or cell-mediated immune responses.

7. Combined *S. suis* MRP2/C05 subunits adjuvanted with either CAF®01 or CDA, failed in providing robust immune responses nor protection in vaccinated animals after intranasal challenge with serovar 2 of *S. suis*, contrary to the results obtained with an autogenous vaccine.

8. Vaccine prototypes containing CAF®01 administered by intramuscular route elicited immune responses capable to clear or modulate the pathogen colonization at mucosal level as observed in the three studies. However, the variability of the achieved immune responses depended on the antigen, fact that will require of further modifications to tailor the immunogenicity towards enhanced and efficient responses.

9. Vaccine prototypes containing soluble CDA mixed with conserved antigens generated a variable or weak immunity, suggesting the need to adjust the form, dose, and routes of administration of the adjuvant and antigens to obtain a consistent immunity.

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List of other publications

As first author

López-Serrano S, Neila-Ibáñez C, Costa-Hurtado M, Mahmmod Y, Martínez-Martínez J, Galindo-Cardiel IJ, Darji A, Rodríguez F, Sibila M, Aragon V. **Sow Vaccination with a Protein Fragment against Virulent *Glaesserella (Haemophilus) parasuis* Modulates Immunity Traits in Their Offspring.** Vaccines (Basel). 2021 May 20;9(5):534. doi: 10.3390/vaccines9050534. PMID: 34065547; PMCID: PMC8160652.

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As contributing author

Blanco-Fuertes M, Correa-Fiz F, López-Serrano S, Sibila M, Aragon V. **Sow vaccination against virulent *Glaesserella parasuis* shapes the nasal microbiota of their offspring.** Sci Rep. 2022 Mar 1;12(1):3357. doi: 10.1038/s41598-022-07382-2. PMID: 35233006; PMCID: PMC8888576.

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Appendix

Chapter III. Immune Responses to Pandemic H1N1 Influenza Virus Infection in Pigs Vaccinated with a Conserved Hemagglutinin HA1 Peptide Adjuvanted with CAF®01 or CDA/αGalCerMPEG

Supplementary table 1. Ventral pictures of the lungs from challenged animals sacrificed at 3 dpi.

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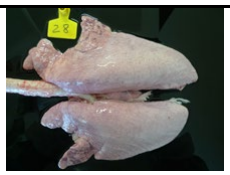
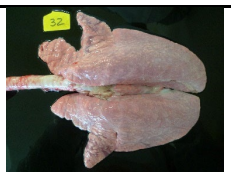
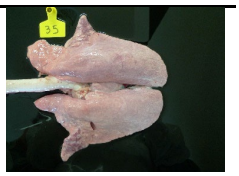
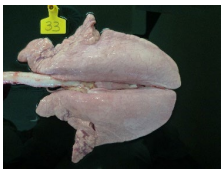
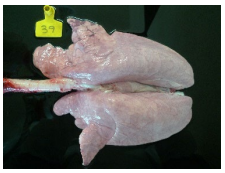
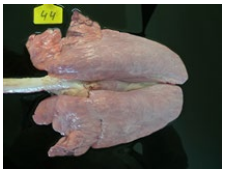
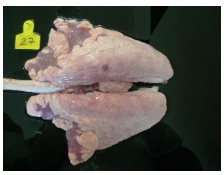
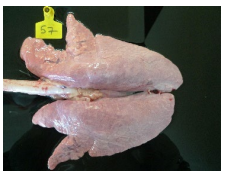
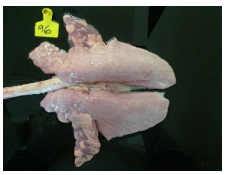
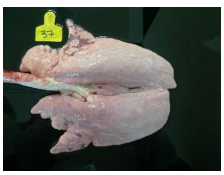
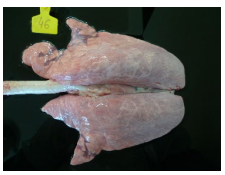
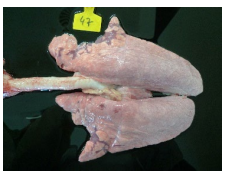
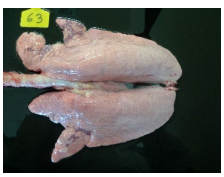
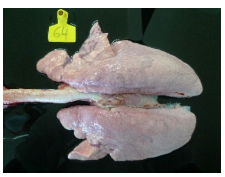
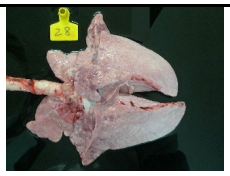
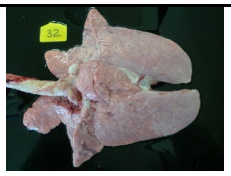
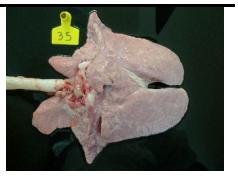
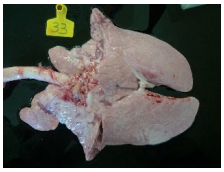
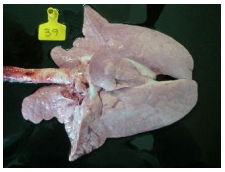
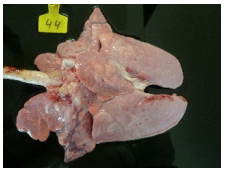
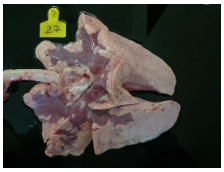
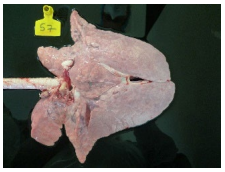
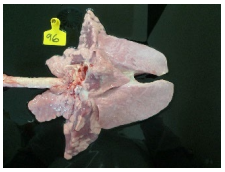
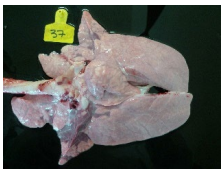
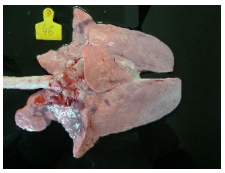
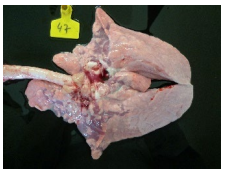
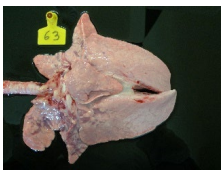
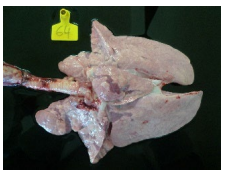
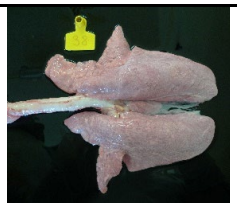
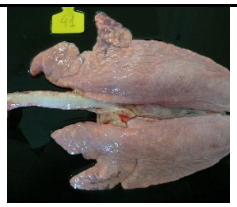
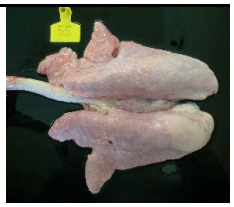
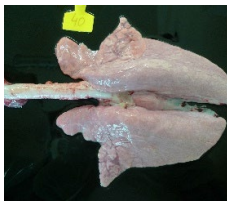
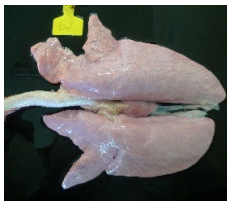
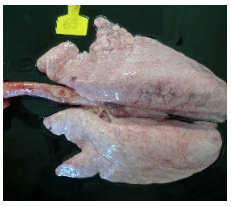
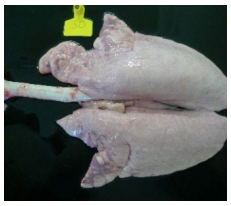
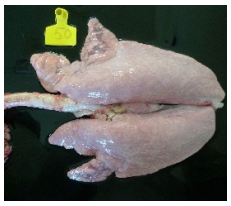
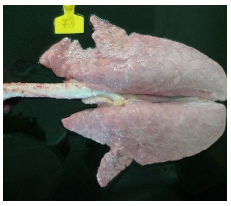
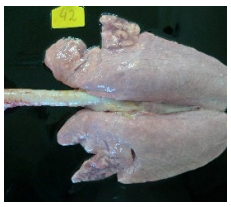
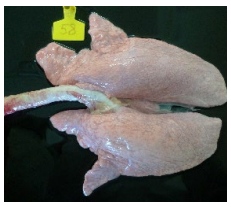
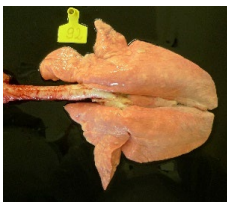
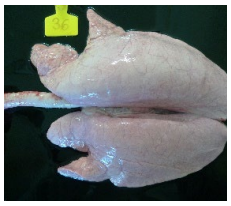
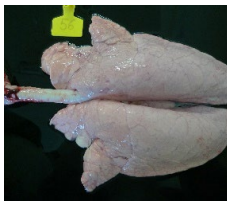
NV/C			
NG34+CAF®01			
NG34+CDA/αGCM			
STIV			
NG34+FA			

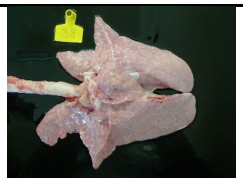
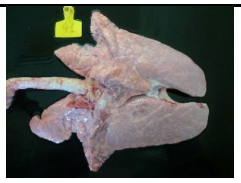
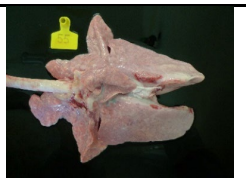
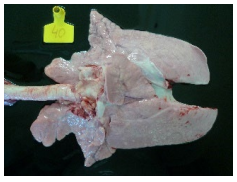
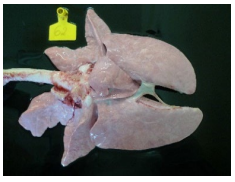
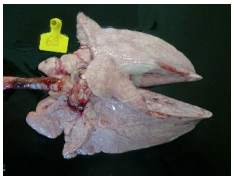
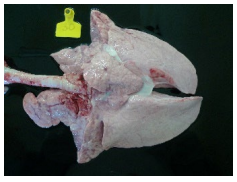
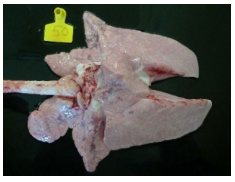
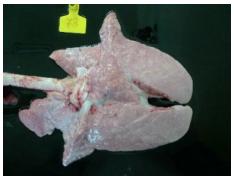
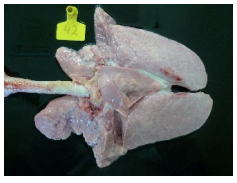
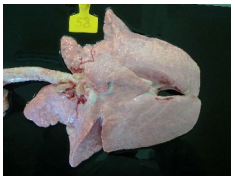
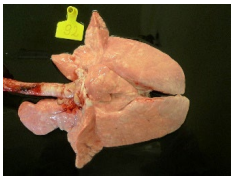
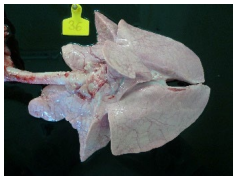
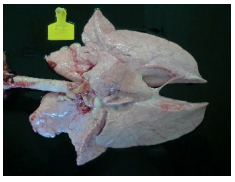
Supplementary table 2. Dorsal pictures of the lungs from challenged animals sacrificed at 3 dpi.

Group	Animals		
NV/C			
NG34+CAF®01			
NG34+CDA/αGCM			
STIV			
NG34+FA			

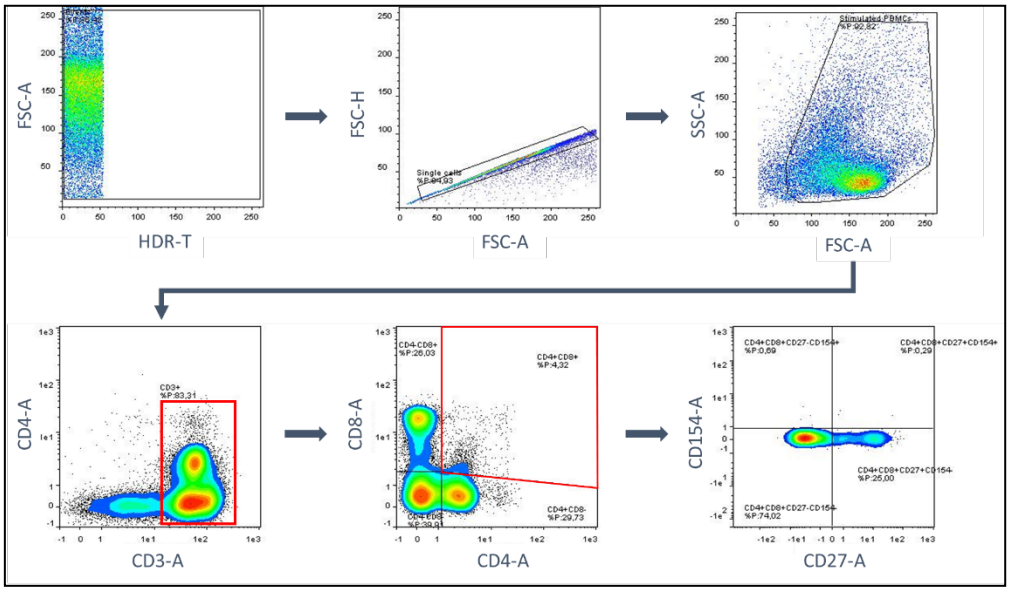
Supplementary table 3. Ventral pictures of the lungs from challenged animals sacrificed at 7 dpi.

Group	Animals		
NV/C			
NG34+CAF®01			
NG34+CDA/αGCM			
STIV			
NG34+FA			

Supplementary table 4. Dorsal pictures of the lungs from challenged animals sacrificed at 7 dpi.

Group	Animals		
NV/C			
NG34+CAF®01			
NG34+CDA/αGCM			
STIV			
NG34+FA			

Chapter IV and V. Immune responses following neonatal vaccination with CAF®01 or CDA adjuvanted conserved fragment F4 against virulent *Glaesserella parasuis* and the effects observed on its natural colonization.

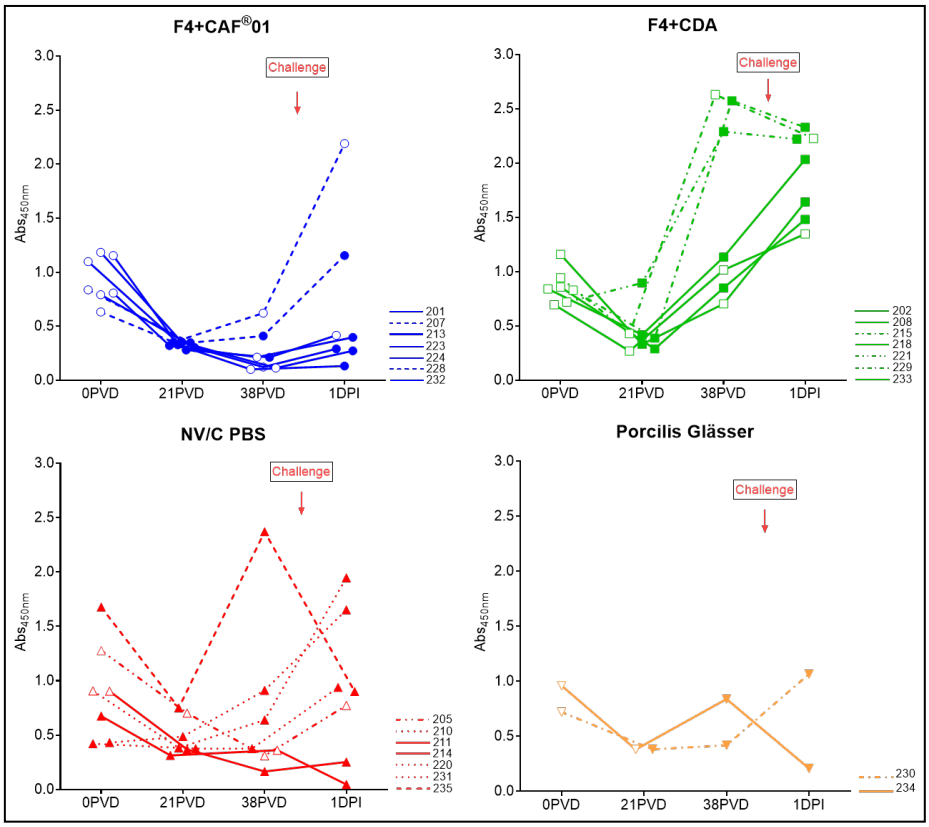


Supplementary figure 1. Gating strategy used for the differentiation of memory T-cell subsets in the experimented animals.

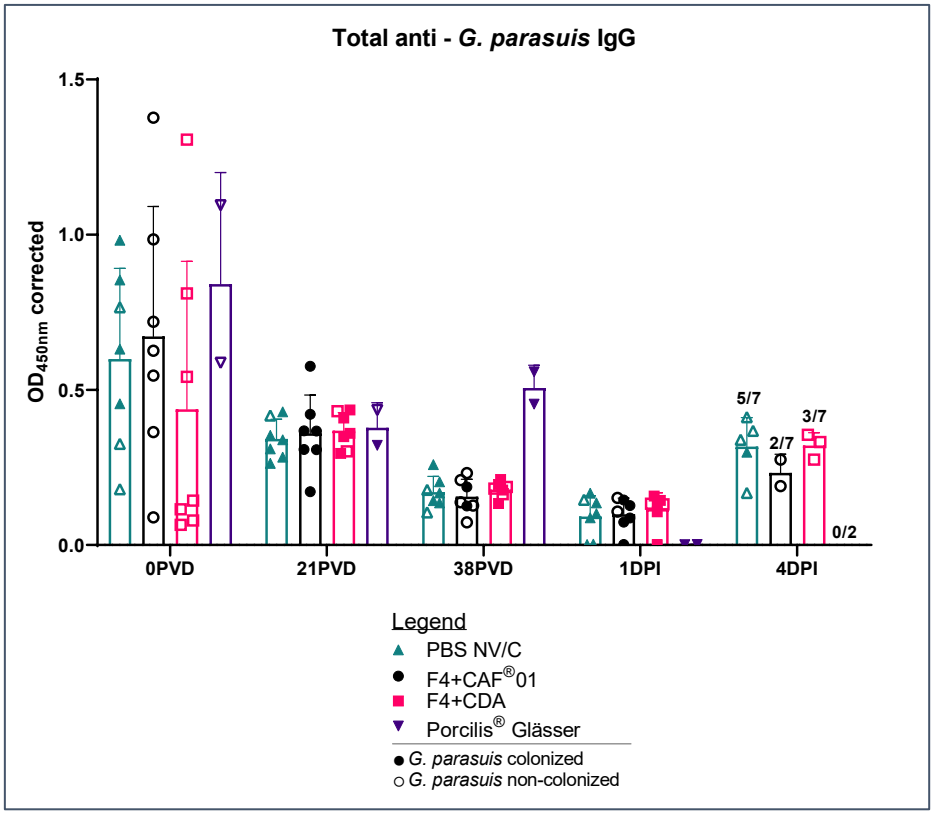
Supplementary table 5. Indicative table of the colonization of the piglets by G. parasuis during the experiment.

Group	Piglet	*G. parasuis* nasal colonization*									
		0 DPV		21 DPV		38 DPV		1 DPI		Necropsies	
		Vir	Nvir	Vir	Nvir	Vir	Nvir	Vir	Nvir	Vir	Nvir
F4+CAF®01	201	-	-	+	-	-	-	+	-	+	-
	207	-	-	+	-	-	-	-	-	-	-
	213	-	-	+	-	+/-	-	-	-	-	-
	223	-	-	+	-	-	-	+	-	+	-
	224	-	-	+	-	-	-	+	-		
	228	-	-	+	+	+/-	-	+	-	+	-
	232	-	-	+	-	-	-	+	-	+	-
F4+CDA	202	-	-	+	-	-	-	+	-	-	-
	208	-	-	+	+	+/-	-	+	-	+	-
	215	-	-	-	-	-	-	-	-		
	218	-	-	-	-	-	-	-	-	-	-
	221	-	-	+	+	+/-	-	+	-	-	-
	229	-	-	+	-	+/-	-	+	-	-	-
	233	-	-	+	-	+/-	-	+	-	-	-
Bacterin	230	-	-	+	-	+/-	-	+	-		
	234	-	-	-	-	+/-	-	+	-	+	-
NV/C	205	-	-	-	-	-	-	-	-	-	-
	210	+	-	+	-	+/-	-	+	-	-	-
	211	+	+	+	-	-	-	+	-		
	214	-	-	+	+	+/-	-	+	-	-	-
	220	-	-	+	-	+/-	-	+	-	-	-
	231	+	-	+	-	+/-	-	+	-		
	235	+	-	+	-	+	-	+	-	+	-

* Nasal colonization: + strong signal, +/- weak signal, - negative



Supplementary figure 2. Anti-F4 IgG individual serologic profiles from the different experimental groups during the study. On the X axis are represented the timepoints where the samples were collected, on the Y axis the absorbance scale. Each point and lines represent the absorbance of each individual piglet diluted to 1/100. F4+CAF®01 group, discontinued lines (207,228): colonized animals which developed an immune response. F4+CDA group; single dotted and discontinued lines (215, 229): sera which presented complement fixation; single dotted and discontinued line (221): enhanced immune response. NV/C PBS group; double dotted discontinued (205): uncolonized animal with maternal antibodies; dotted lines (210, 220, 231): colonized animals which developed immune response; bold lines (211, 214): animals without immune response. Porcilis Glässer group; discontinued dotted line (230) animal that presented fixation to the complement.



Supplementary figure 3. Total anti – G. parasuis antibody levels measured throughout the second study using Ingezim-Haemophilus ELISA (Ingenasa, Madrid, Spain). Individual levels are represented in symbols. Filled symbols represent the piglets colonized by serovar 4 of G, parasuis whereas empty symbols represent uncolonized animals. Averages and standard deviations of each group are also plotted in bars.

Supplementary table 6. Association of IFN-γ secretion 21 days after the first vaccination with the sow of origin and their anti-F4 IgG level.

Variable	Level	Estimate	95% CI	*p* value
Anti-F4 IgG in sow	---	0.142	0.019 - 0.265	0.02556
Colonization with virulent *G.parasuis* at 38DPV	0	Ref	Ref	0.0021
	1	22.00	-17.95 - 61.95	
Sow origin	Sow 1	Ref	Ref	1.36e-08
	Sow 2	559.33	477.77 - 640.89	
	Sow 3	-9.33	-72.50 - 53.83	
	Sow 4	0.333	-56.85 - 57.52	
	Sow 5	-4.66	-85.68 - 76.34	
	Sow 6	-39.66	-101.06 - 21.72	
	Sow 7	-18.00	-81.17 - 45.17	
	Sow 8	-38.16	-105.004 - 28.67	

Supplementary table 7. Association of obtained T helper cells (CD4+) upon the in vitro stimulation and sow traits using multivariable model.

Lymphocyte subset / Stimulus	Variable	Level	Estimate	95% CI	*p* value
T helper cells (CD4+) Nagasaki	Sow parity	3	Ref	Ref	0.0236
		4	-8.184	-13.85 - (-2.52)	
		5	-2.907	-10.92 - 5.11	
	Anti-*G.parasuis* antibody ratio in sow	Doubtful	Ref	Ref	0.0382
		Positive	10.28	2.31 - 18.25	
		Negative	5.52	-0.53 - 11.56	
	Sow origin	Sow 1	Ref	Ref	0.039
		Sow 2	-1.510	-14.77 - 11.75	
		Sow 3	-1.300	-10.68 - 8.077	
		Sow 4	-9.202	-17.97 - (-0.43)	
		Sow 5	11.740	-1.52 - 25.00	
		Sow 6	5.2900	-3.48 - 14.06	
		Sow 7	0.636	-8.7 - 10.01	
		Sow 8	0.0175	-8.75 - 8.78	
T helper cells (CD4+) F4	Sow parity	3	Ref	Ref	0.02301
		4	-8.90875	-15.05 (-2.76)	
		5	-3.05000	-11.7 - 5.64	
	Anti-*G.parasuis* antibody ratio in sow	Doubtful	Ref	Ref	0.04173
		Positive	10.67	1.98 - 19.37	
		Negative	6.54	-0.06 - 13.13	

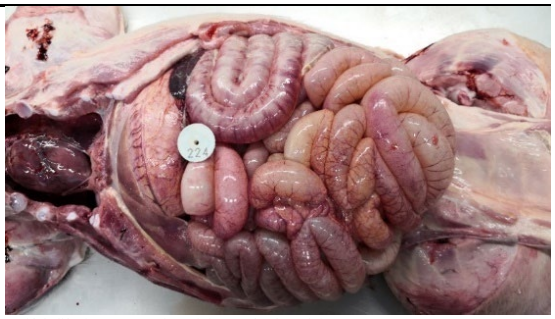
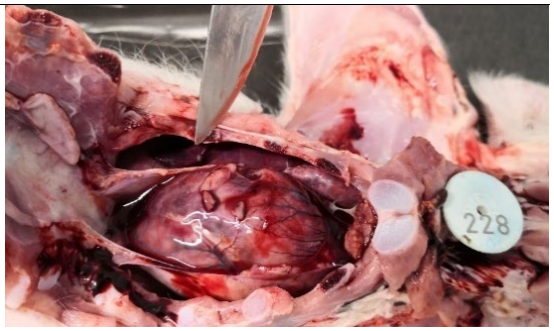
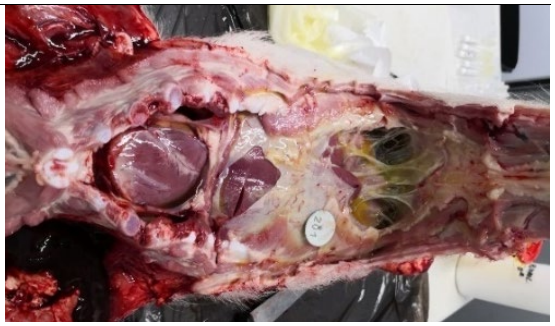
Supplementary table 8. Association of Memory T cells subsets upon the in vitro stimulation with F4 and sow traits using multivariable model.

Lymphocyte subset / Stimulus	Variable	Level	Estimate	95% CI	*p* value
Memory T cells (CD4+CD8+) F4	Sow parity	3	Ref	Ref	0.0256
		4	-1.8912	-3.22 – (-0.56)	
		5	-0.9908	-2.87 – 0.89	
Central Memory T cells (CD4+CD8+CD27+) F4	Sow parity	3	Ref	Ref	0.02215
		4	-0.4995	-0.849 - (-0.149)	
		5	-0.05083	-0.546 - 0.444	
	Anti-*G.parasuis* antibody ratio in sow	Doubtful	Ref	Ref	0.005704
		Positive	0.79178	0.342 - 1.241	
		Negative	0.2626	-0.078- 0.60	
	Sow origin	Sow 1	Ref	Ref	0.02478
		Sow 2	-0.460	-1.254 - 0.334	
		Sow 3	-0.183	-0.745 - 0.377	
		Sow 4	-0.645	-1.170 - (-0.12)	
		Sow 5	0.140	-0.65 - 0.933	
		Sow 6	0.387	-0.138 - 0.912	
		Sow 7	-0.083	-0.64 - 0.478	
		Sow 8	-0.208	-0.73 - 0.317	
Effector Memory T cells (CD4+CD8+CD27-) F4	Sow parity	3	Ref	Ref	0.04875
		4	-1.374	-2.481 - (-0.27)	
		5	-0.980	-2.55 - 0.59	

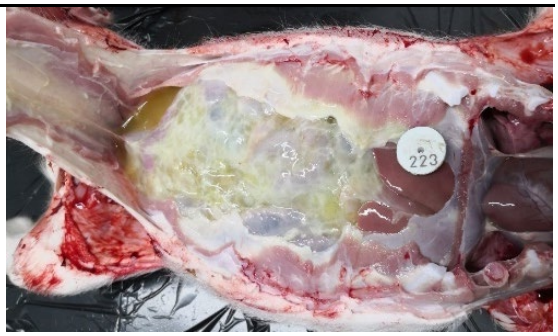
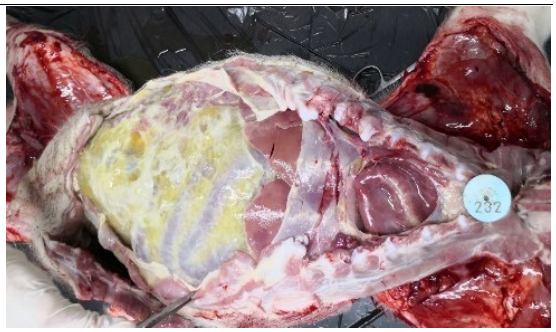
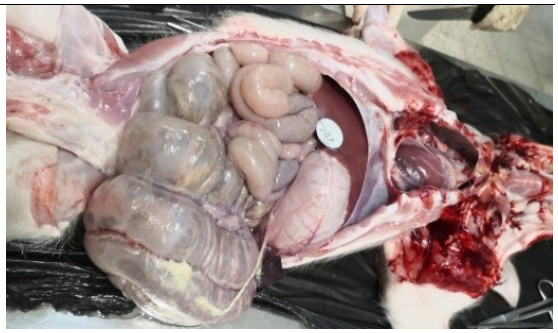
Supplementary table 9. Final model with the association of secreted cytokines upon different in vitro stimulus and predictor variables using multivariable model.

Cytokine / Stimulus	Variable	Level	Estimate	95% CI	*p* value
IFN-α Nagasaki	Colonization with virulent *G.parasuis* at 38DPV	0	Ref	Ref	0.066
		1	2.28	-0.174 - 4.73	
	Sow origin	Sow 1	Ref	Ref	0.70670
		Sow 2	1.90	-3.064 - 6.87	
		Sow 3	-0.87	-4.74 - 2.99	
		Sow 4	1.38	-2.12 - 4.889	
		Sow 5	0.93	-4.03 - 5.90	
		Sow 6	0.93	-5.56 - 1.96	
		Sow 7	-0.91	-4.78 - 2.966	
		Sow 8	-1.59	-5.69 - 2.50	
TGF-β Nagasaki	Anti-F4 IgG in sow	---	0.1227605	0.017 - 0.23	0.025
	Colonization with virulent *G.parasuis* at 38DPV	0	Ref	Ref	0.054
		1	-84.05	-169.79 - 1.69	
TGF-β F4	Anti-F4 IgG in sow	---	0.107909	7.2e-03 - 0.21	0.03691
	Anti-*G.parasuis* antibodies in sow	Doubtful	Ref	Ref	0.04577
		Positive	-112.6958	-227.80 - 2.41	
		Negative	23.24586	-64.09 - 110.59	

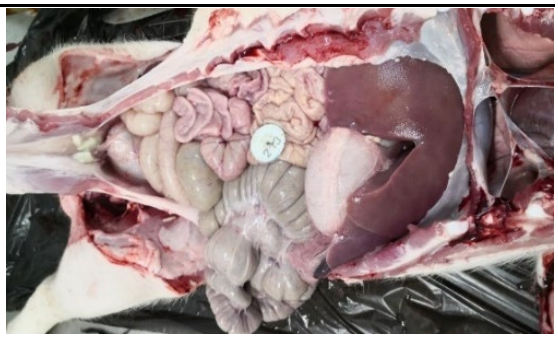
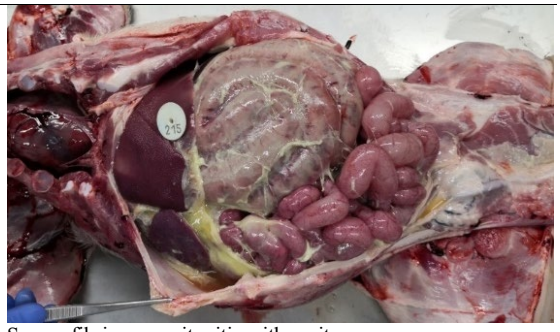
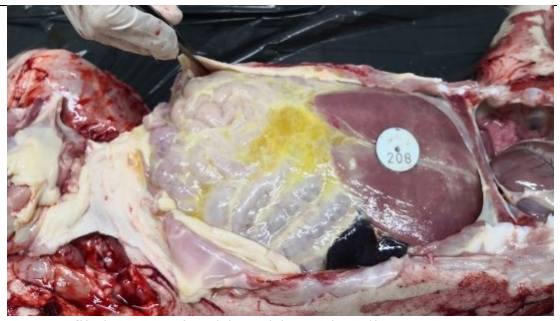
Supplementary table 10. Post-mortem pictures of the experimented animals collected during the necropsies. The animals within a group are ordered according to their termination day.

Group	Pig ID	Termination day	Pictures
F4+CAF01	224	1dpi	 Mild fibrinous peritonitis and pleuritis.
F4+CAF01	228	1dpi	 Severe peritonitis and pleuritis with liquid in pericardium.
F4+CAF01	201	2dpi	 Severe fibrinous peritonitis with ascites and adherences. Severe fibrinous pleuritis with adherences and pleural liquid. Hidropericardium.

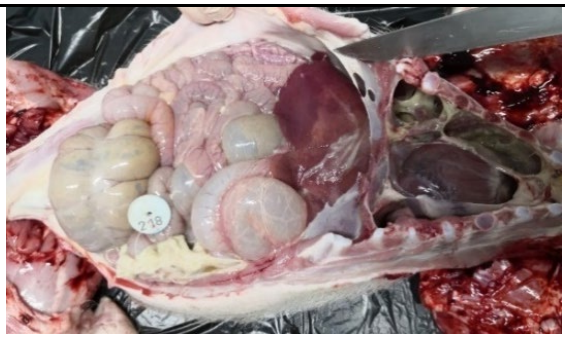
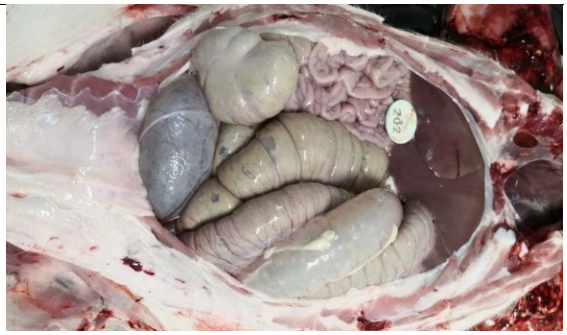
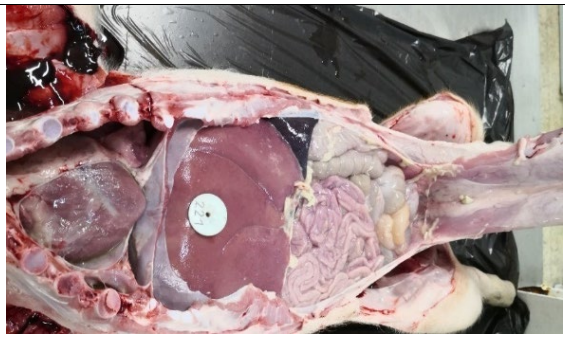
Supplementary table 10 (Continuation 1)

Group	Pig ID	Termination day	Pictures
F4+CAF01	223	2dpi	 Severe fibrinous peritonitis.
F4+CAF01	232	2dpi	 Severe fibrinous peritonitis with ascites, pleuritis and pericarditis with hidropericardium.
F4+CAF01	207	4dpi	 Moderate fibrinous peritonitis. Mild fibrinous pleuritis with adherences and pericarditis with Hidropericardium.

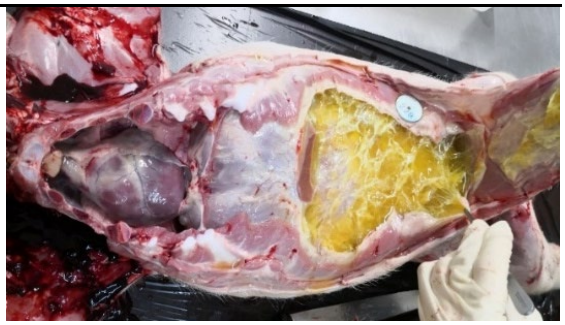
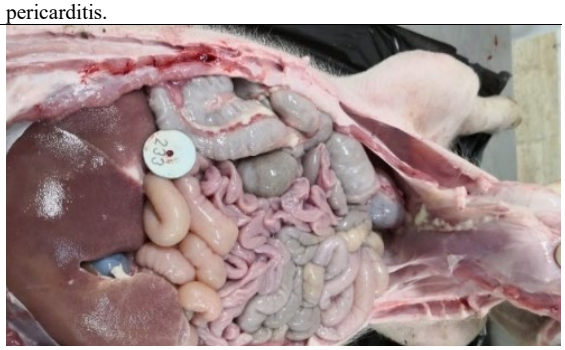
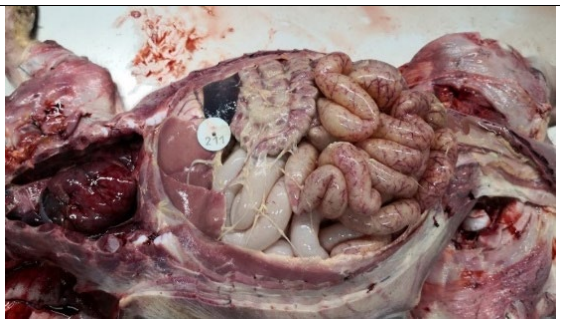
Supplementary table 10 (Continuation 2)

Group	Pig ID	Termination day	Pictures
F4+CAF01	213	4dpi	 Mild fibrinous peritonitis localized in the inoculation site. Hidropericardium.
F4+CDA	215	1dpi	 Severe fibrinous peritonitis with ascites.
F4+CDA	208	2dpi	 Severe fibrinous peritonitis. Hidropericardium.

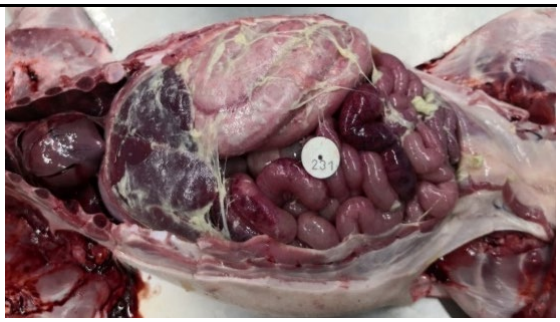
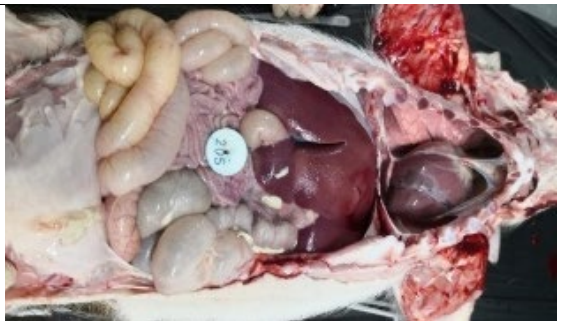
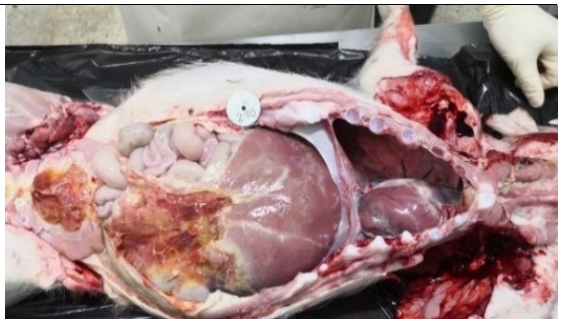
Supplementary table 10 (Continuation 3)

Group	Pig ID	Termination day	Pictures
F4+CDA	218	2dpi	 Severe fibrinous peritonitis and pleuritis.
F4+CDA	202	4dpi	 Mild fibrinous peritonitis localized in the inoculation site.
F4+CDA	221	4dpi	 Moderate fibrinous peritonitis, pleuritis and pericarditis with hidropericardium.

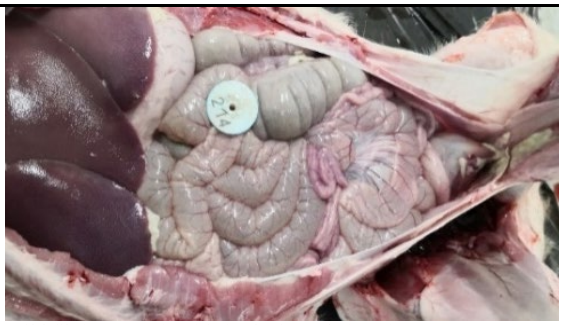
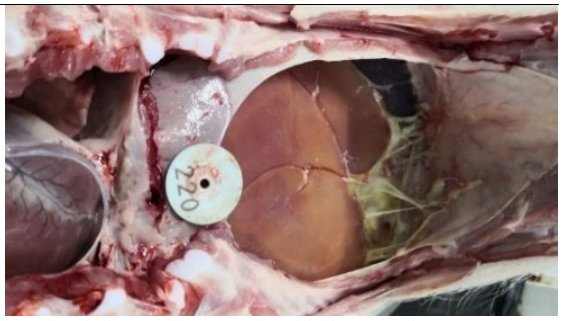
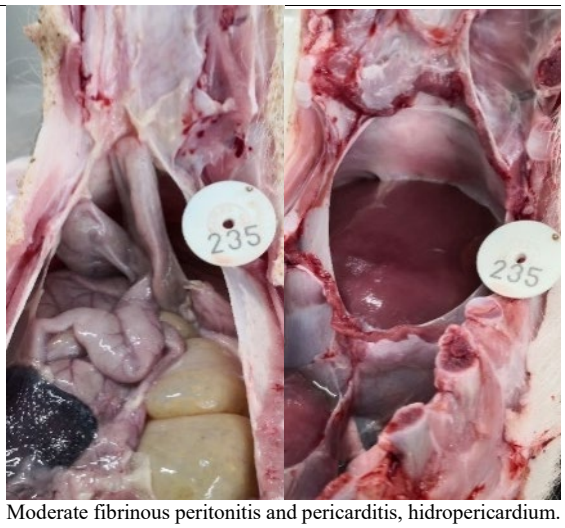
Supplementary table 10 (Continuation 4)

Group	Pig ID	Termination day	Pictures
F4+CDA	229	4dpi	 Severe fibrinous peritonitis with ascites, mild fibrinous pericarditis.
F4+CDA	233	4dpi	 Mild fibrinous peritonitis localized in the inoculation site.
NV/C (PBS)	211	1dpi	 Moderate fibrinous peritonitis with ascites. Mild fibrinous pleuritis**.**

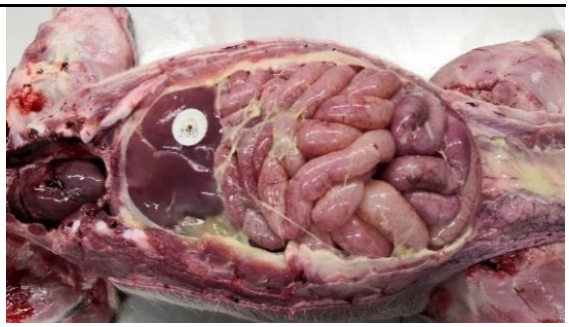
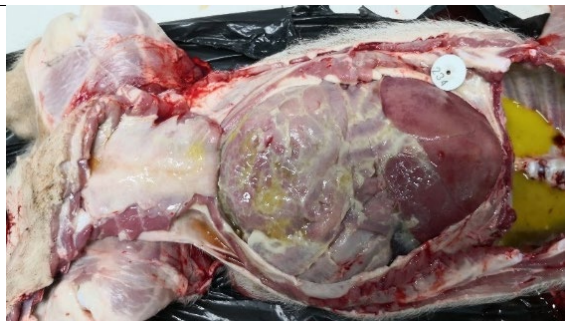
Supplementary table 10 (Continuation 5)

Group	Pig ID	Termination day	Pictures
NV/C (PBS)	231	1dpi	 Severe fibrinous peritonitis with ascites.
NV/C (PBS)	205	4dpi	 Mild fibrinous peritonitis. Moderate hidropericardium.
NV/C (PBS)	210	4dpi	 Fibrinous peritonitis with minimum ascites, hidropericardium.

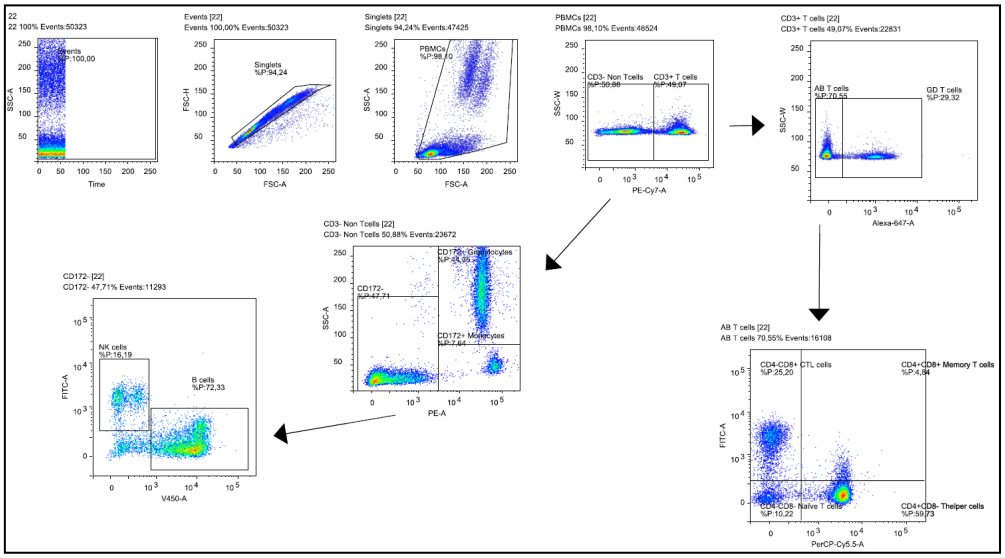
Supplementary table 10 (Continuation 6)

Group	Pig ID	Termination day	Pictures
NV/C (PBS)	214	4dpi	 Mild fibrinous peritonitis in the inoculation site.
NV/C (PBS)	220	4dpi	 Localized fibrinous peritonitis, mild fibrinous pleuritis.
NV/C (PBS)	235	4dpi	 Moderate fibrinous peritonitis and pericarditis, hidropericardium.

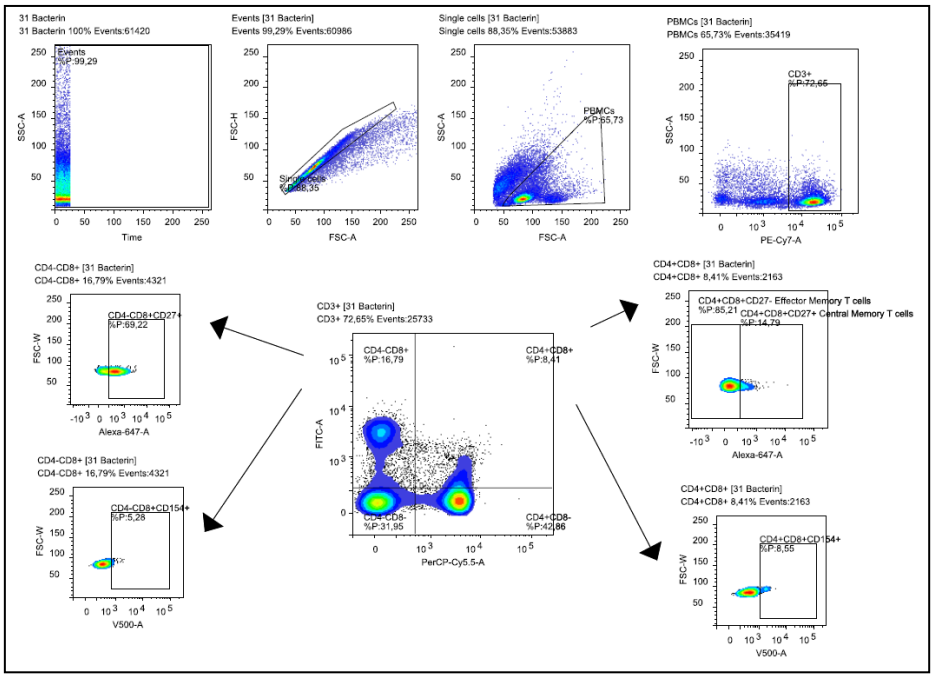
Supplementary table 10 (Continuation 7)

Group	Pig ID	Termination day	Pictures
Bacterin vaccine	230	1dpi	 Severe fibrinous peritonitis.
Bacterin vaccine	234	2dpi	 Severe fibrinous peritonitis with ascites, mild fibrinous pleuritis.

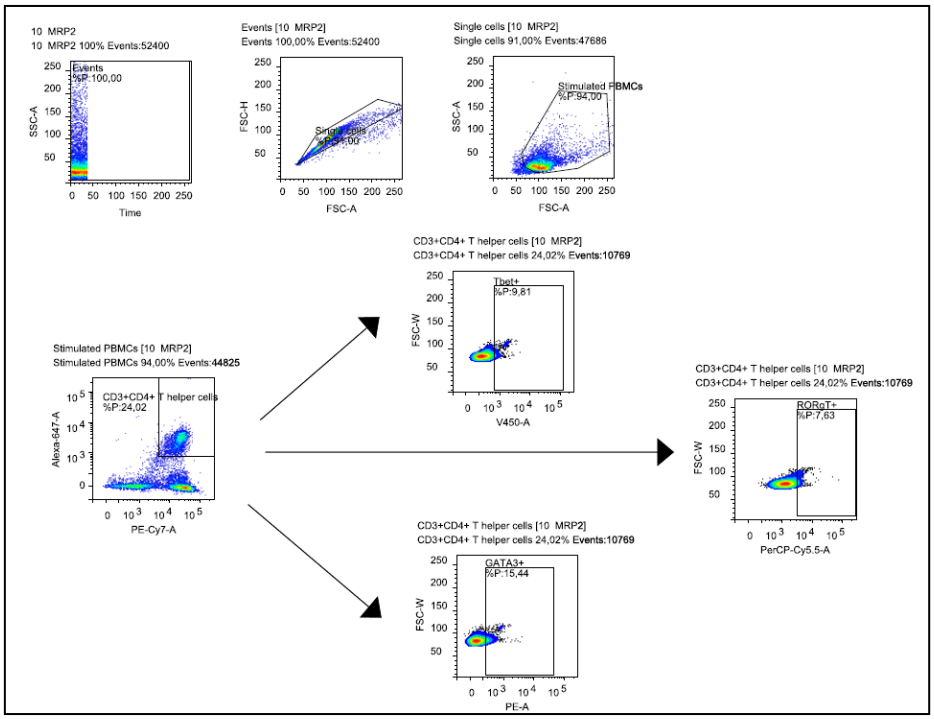
Chapter VI. Immune responses in new-born piglets after immunization with *Streptococcus suis* surface-conserved antigens combined with CAF®01 or CDA



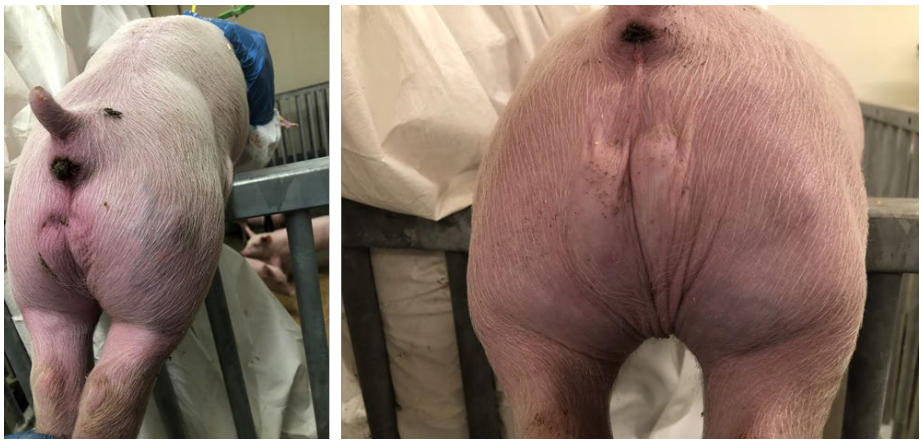
Supplementary figure 4. Gating strategy applied for the analysis of PBMC cell populations by surface staining CD3, CD4, CD8, CD21, CD172 and γδ-TCR.



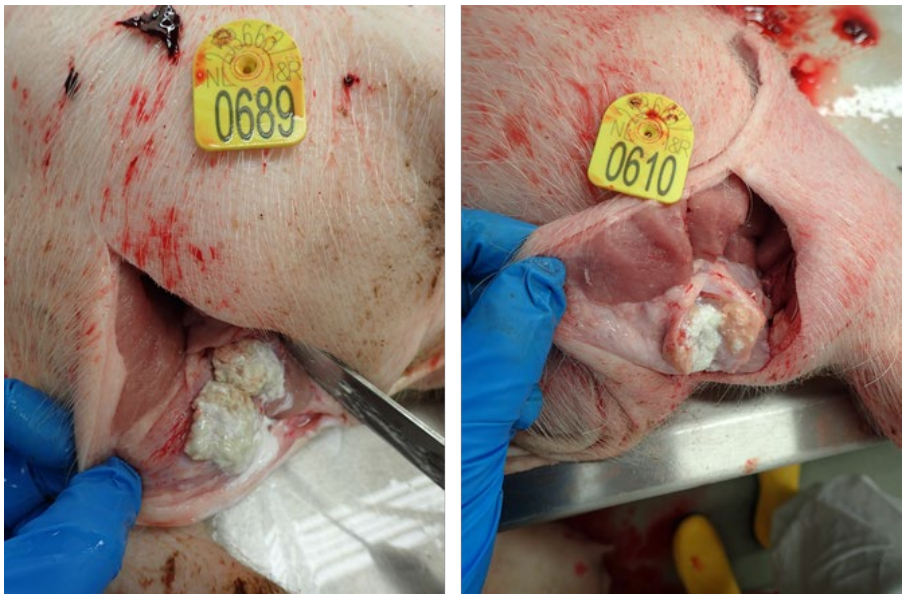
Supplementary figure 5. Gating strategy for the analysis of specific Memory and reactive T-cell subsets from in vitro stimulated PBMCs by staining of surface markers CD3, CD4, CD8, CD27, and CD154.



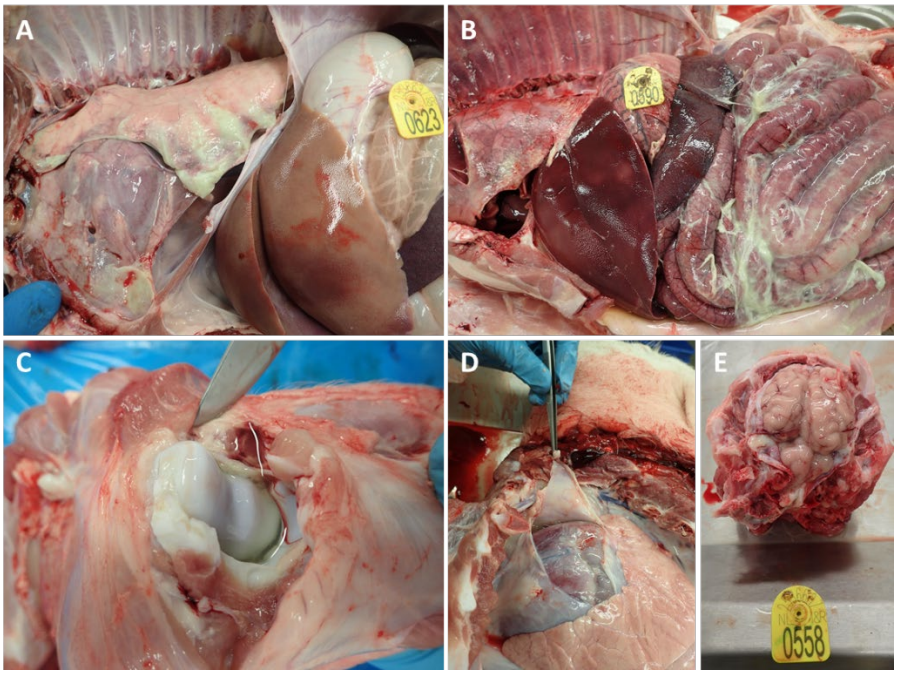
Supplementary figure 6. Gating strategy for the analysis of specific Th cell subsets from in vitro stimulated PBMCs labelling master transcription factors Tbet, GATA3 and RORγT.



Supplementary figure 7. Examples of the swelling and vascularization observed in the hind legs of the animals vaccinated with SS10/Specol® formulation (Pictures courtesy of Sandra Vreman).



Supplementary figure 8. Dissection at post-mortem examination of the hind leg granulomatous-like lesions observed in SS10/Specol® vaccinated piglets (Pictures courtesy of Sandra Vreman).



Supplementary figure 9. Examples of lesions caused by S. suis infection in the animals. (A) Pleuritis. (B) Peritonitis. (C) Arthritis. (D) Pericarditis. (E) Meningitis (Pictures courtesy of Sandra Vreman).