Matthijs *et al. Vet Res* (2019) 50:91
https://doi.org/10.1186/s13567-019-0709-0

VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Efficacy of three innovative bacterin vaccines against experimental infection with *Mycoplasma hyopneumoniae*

Anneleen Marguerite Filip Matthijs[1*], Gaël Auray[2,3], Filip Boyen[4], Alexandra Schoos[1], Annelies Michiels[1], Obdulio García-Nicolás[2,3], Güliz Tuba Barut[2,3], Christophe Barnier-Quer[5], Virginie Jakob[5], Nicolas Collin[5], Bert Devriendt[6], Artur Summerfield[2,3], Freddy Haesebrouck[4] and Dominiek Maes[1]

Abstract

New vaccine formulations that include novel strains of *Mycoplasma hyopneumoniae* and innovative adjuvants designed to induce cellular immunity could improve vaccine efficacy against this pathogen. The aim of this experimental study was to assess the efficacy of three experimental bacterin formulations based on *M. hyopneumoniae* field strain F7.2C which were able to induce cellular immunity. The formulations included a cationic liposome formulation with the Mincle receptor ligand trehalose 6,6-dibehenate (Lipo_DDA:TDB), a squalene-in-water emulsion with Toll-like receptor (TLR) ligands targeting TLR1/2, TLR7/8 and TLR9 (SWE_TLR), and a poly(lactic-*co*-glycolic acid) micro-particle formulation with the same TLR ligands (PLGA_TLR). Four groups of 12 *M. hyopneumoniae*-free piglets were primo- (day (D) 0; 39 days of age) and booster vaccinated (D14) intramuscularly with either one of the three experimental bacterin formulations or PBS. The pigs were endotracheally inoculated with a highly and low virulent *M. hyopneumoniae* strain on D28 and D29, respectively, and euthanized on D56. The main efficacy parameters were: respiratory disease score (RDS; daily), macroscopic lung lesion score (D56) and log copies *M. hyopneumoniae* DNA determined with qPCR on bronchoalveolar lavage (BAL) fluid (D42, D56). All formulations were able to reduce clinical symptoms, lung lesions and the *M. hyopneumoniae* DNA load in the lung, with formulation SWE_TLR being the most effective ($RDS_{D28–D56}$ −61.90%, macroscopic lung lesions −88.38%, *M. hyopneumoniae* DNA load in BAL fluid (D42) −67.28%). Further experiments raised under field conditions are needed to confirm these results and to assess the effect of the vaccines on performance parameters.

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (EP) in pigs. This chronic respiratory disease is responsible for major economic losses in pig producing countries all over the world due to reduced performance and increased medication use [1, 2].

Together with biosecurity measurements, management practices and medication, commercial bacterin vaccines are used worldwide to control EP [1]. They are mostly constituted of inactivated whole cells of the J strain, a low virulent *M. hyopneumoniae* field strain isolated in the UK in the 1950s [3], and adjuvants such as aluminium hydroxide, carbopol, mineral oil or biodegradable oil [4]. Vaccination reduces clinical symptoms, lung lesions and performance losses [5, 6]. However, current commercial vaccines neither prevent colonisation of the pathogen, nor the development of clinical signs and lung lesions [7]. Also, their effect on disease transmission is only limited [8–10]. Moreover, the beneficial effects of vaccination are known to vary between herds [4], which may be partially due to pathogenic and antigenic differences between the strains circulating on the farms and the vaccine strain [11].

*Correspondence: Anneleen.Matthijs@ugent.be
[1] Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
Full list of author information is available at the end of the article

While serum antibodies are not correlated with protection against EP [12], the role of mucosal antibodies (immunoglobulin (Ig) A) is still unclear. According to Thacker et al. [13], mucosal IgA could prevent colonisation of the microorganism in the respiratory tract of the pig. Cell-mediated immune responses, more specifically T helper (Th) 1, Th17 and $CD8^+$ T cell responses, are considered to play a major role in protective immunity against *M. hyopneumoniae* infections [13–16]. T helper 1 cells are considered to contribute to protection against *Mycoplasma* infections by activating macrophage killing [14], while Th17 cells protect the lung mucosa by elevating secretory IgA levels [17] and recruiting neutrophils for pathogen clearance [18]. $CD8^+$ T cells, on the other hand, might dampen the excessive pro-inflammatory Th responses that induce lung lesions and clinical disease [19].

Research into novel vaccine formulations that may offer a better protection against *M. hyopneumoniae* is constantly ongoing. An overview of the different experimental *M. hyopneumoniae* vaccines already showed that most of them are based on recombinant proteins of *M. hyopneumoniae* and were evaluated in mice [4]. Merely a few of them were tested in challenge experiments in pigs. Also, none of these formulations were able to offer total protection or similar protection as the commercially available vaccines, despite their often promising immunizing properties [4, 20].

In a previous study [21], the safety and the immune responses of five innovative bacterin formulations were evaluated in pigs. All formulations were based on *M. hyopneumoniae* strain F7.2C, a highly virulent field strain shown to be antigenically different from the J strain [22, 23], and contained adjuvants specifically designed to promote cellular immunity. Three promising vaccine formulations were identified based on their ability to induce potent *M. hyopneumoniae*-specific T cell responses. These included a micro-particle and an oil-in-water formulation to deliver a cocktail of Toll-like receptor (TLR) 1/2, TLR9 and TLR7/8 ligands, and a cationic liposomal formulation to deliver a Mincle ligand. The liposomal formulation was able to induce strong Th1 and $CD8^+$ T cell responses, while the oil-in-water formulation induced a strong Th1 response and a moderate $CD8^+$ T cell response. The micro-particle formulation had the unique ability of inducing a potent Th17 response. Therefore, the aim of this study was to assess the protective efficacy of these three innovative bacterin formulations against experimental infection with two *M. hyopneumoniae* field strains. The main efficacy parameters were respiratory disease score (RDS), macroscopic lung lesion score and log copies *M. hyopneumoniae* DNA in bronchoalveolar lavage (BAL) fluid. Additionally, microscopic lung lesions, *M. hyopneumoniae*-specific local and systemic antibodies, *M. hyopneumoniae*-specific systemic T cell responses and cytokine responses in BAL fluid were assessed.

This study demonstrated the potential of innovative *M. hyopneumoniae* bacterin formulations and identified promising vaccine candidates for further exploration.

Materials and methods

Vaccines and adjuvants

Three adjuvant formulations were developed based on the association of particle-based delivery systems (liposomes, poly(lactic-*co*-glycolic acid) (PLGA) microparticles and a squalene-in-water emulsion (SWE)) with different immune stimulators. These included the Mincle agonist trehalose 6,6-dibehenate (Avanti, Alabaster, AL, USA) and a combination of TLR ligands: TLR1/2 ligand Pam3Cys-SK4 (PAM; EMC Microcollections, Tübingen, Germany), TLR9 ligand CpG oligodeoxynucleotides SL03 (CpG; Eurofins Genomics, Les Ulis, France) and TLR7/8 ligand resiquimod (Chemdea, Ridgewood, NJ, USA). The selection of the TLR ligands was based on their ability to activate porcine antigen presenting cells [24–26].

Trehalose 6,6-dibehenate (TDB) was combined with dimethyl dioctadecylammonium (DDA) bromide with the thin lipid film method [27] and followed by extrusion to form the cationic liposome formulation Lipo_DDA:TDB. Cationic poly(lactic-*co*-glycolic acid) micro-particles (combined to ethylaminoethyl-dextran) were produced with the double emulsion (W/O/W) methods [28]. Pam3Cys-SK4 and resiquimod were encapsulated into the particles and CpG was subsequently adsorbed onto their surface for the PLGA_TLR formulation. The oil-in-water formulation SWE_TLR was obtained by mixing SWE (a squalene-based formulation developed and produced by the Vaccine Formulation Laboratory, and composed of 3.9% (w/v) squalene, 0.5% (w/v) Tween 80 and 0.5% (w/v) Span 85 [29]) with the immune stimulators PAM, CpG and resiquimod.

The vaccine strain *M. hyopneumoniae* F7.2C was grown in modified Friis medium [30] for 5 days at 35 ± 2 °C. The culture, containing 5×10^8 colour changing units (CCU)/mL, was inactivated by incubation with 4 mM binary ethyleneimine (BEI) under agitation at 35 ± 2 °C for 24 h. Subsequently, the BEI was neutralised by incubating the inactivated culture with 4 mM sodium thiosulfate under agitation at 35 ± 2 °C for 24 h. Inactivated bacteria were pelleted at 15 000 *g* for 40 min at 4 °C and washed three times in 50 mL sterile phosphate buffered saline (PBS). The final pellet was resuspended in sterile PBS and mixed with the different adjuvants. The composition of each experimental vaccine is given in Table 1.

Table 1 Composition of the experimental *M. hyopneumoniae* bacterins and their route of administration

Vaccine formulation	Dose (mL)	Delivery system	Immune-stimulators (µg/dose)	Antigen dose (CCU/dose)	Administration route (primo and booster)
Lipo_DDA:TDB	2	DDA liposomes	TDB (500)	10^9	IM
PLGA_TLR		PLGA micro-particles (combined to ethylaminoethyl-dextran)	Pam3Cys-SK4/CpG ODN SL03/resiquimod (80/80/80)		
SWE_TLR		Squalene-in-water emulsion			

CCU: colour changing units, IM: intramuscular, PLGA: poly(lactic-*co*-glycolic acid), DDA: dimethyl dioctadecylammonium, TDB: trehalose 6,6′-dibehenate.

Study animals and experimental design

The study was performed after approval by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2017/120). Fifty-three *M. hyopneumoniae*-free Rattlerlow-Seghers piglets (RA-SE Genetics NV, Ooigem, Belgium) were enrolled in the study. All animals originated from a herd that has been *M. hyopneumoniae*-free for many years based on repeated serological testing, nested PCR testing on tracheobronchial swabs, and absence of clinical signs and pneumonia lesions at slaughter. The piglets arrived at the experimental facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium at 32 days of age. They were housed in stables with absolute air filters for impending particles (HEPA U15) on both incoming and outgoing ventilation shafts and fed ad libitum with a non-antimicrobial-supplemented diet. On the day of arrival (D-7), the piglets were randomly allocated into three vaccination groups and a PBS-injected control group (PCG) of 12 piglets each. Five pigs were included as a non-challenge control group (NCG). After an acclimatization period of 1 week, the pigs of the vaccination groups were prime-boost vaccinated intramuscularly (IM) at day (D) 0 and D14 of the study with 2 mL of experimental bacterin. The pigs of the PCG and NCG received 2 mL sterile PBS IM at both vaccination days. The pigs of the vaccinated groups and the PCG were experimentally infected by endotracheal inoculation of the highly virulent *M. hyopneumoniae* strain F7.2C (7 mL culture medium containing 10^7 CCU/mL) on D28 and the low virulent strain F1.12A (7 mL culture medium containing 10^7 CCU/mL) on D29 [31]. The pigs of the NCG were endotracheally inoculated with sterile culture medium on both challenge days. For the inoculations, the pigs were anesthetized by administering 0.22 mL/kg body weight of a mixture of Zoletil 100® (Virbac, Louvain la Neuve, Belgium) and Xyl-M® 2% (VMD, Arendonk, Belgium) IM. Four weeks after challenge infection (D56), all pigs were euthanized by exsanguination after deep anaesthesia with 0.3 mL/kg of the same mixture injected IM.

Clinical and performance parameters

The pigs were observed daily between 8 and 10 a.m. for at least 20 min from D-6 until D56 of the study. For each pig, remarkable clinical findings (loss of appetite, diarrhoea, dyspnoea, depression, lameness) were recorded and the severity of coughing was assessed by means of a RDS [32]. The scoring could range from 0 to 6 with 0 = no coughing, 1 = mild coughing after an encouraged move, 2 = mild coughing in rest, 3 = moderate coughing after an encouraged move, 4 = moderate coughing in rest, 5 severe coughing after an encouraged move, 6 = severe coughing in rest.

On the day of primo-vaccination (D0), challenge (D28) and euthanasia, the pigs were weighed, and the average daily gain (ADG; g/pig/day) was calculated from D0–28, D28–56 and D0–56 [33].

Macroscopic and microscopic lung lesions

At necropsy (D56), the lungs were removed and scored for macroscopic *Mycoplasma*-like lesions according to Hannan et al. [34]. The score could range from 0 (no lesions) to 35 (entire lung affected).

From each pig, samples from the left apical, cardiac and diaphragmatic lung lobe were collected for histopathological examination. If lesions were present, samples were taken from the border of the lesion. Each sample was scored for the degree of peribronchiolar and perivascular lymphohistiocytic infiltration, as well as nodule formation, using light microscopy. A score system ranging from 1 to 5 was used, with 1 = limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates, 2 = light to moderate infiltrates with mild diffuse cellular exudates into airways, 3–4–5 = respectively mild, moderate and severe lesions characteristic of bronchointerstitial pneumonia, centred around bronchioles but extending to the interstitium, with lymphofollicular infiltration and mixed inflammatory cell exudates [35]. Scores 1 and 2 are considered to be not related to *M. hyopneumoniae*, while scores 3–5 are suggestive for an infection with *M. hyopneumoniae*.

The percentage of lung area occupied by air (% air) was assessed using an automated image analysis system (Leica Application Suite AF Lite (Diegem, Belgium) and ImageJ (Bethesda Softworks, Rockville, MD, USA) [31]).

Quantitative PCR for *M. hyopneumoniae* DNA and routine bacteriological culture on bronchoalveolar lavage fluid

Two weeks after challenge infection (D42), bronchoalveolar lavage fluid was collected from each pig by inserting a catheter (Portex® Dog Catheter with Female Luer Mount, Smiths Medical International Ltd., Kent, UK) in the trachea and flushing the lungs with 20 mL sterile PBS [31]. At necropsy (D56), BAL fluid was collected from the right lung by flushing the head bronchus with 20 mL sterile PBS as previously described [36]. Deoxyribonucleic acid was extracted from the BAL fluid using a commercial kit (DNeasy® Blood & Tissue kit, Qiagen, Venlo, The Netherlands) and a quantitative PCR (qPCR) was performed according to Marois et al. [37] to measure the number of *M. hyopneumoniae* organisms. The threshold values were converted to the number of organisms using a tenfold dilution series of *M. hyopneumoniae* F7.2C DNA. Values below the highest dilution (1.50×10^1/mL; 1.18 log copies/mL) were considered as negative.

From each pig, 10 µL of BAL fluid collected at D56 was inoculated on a Columbia agar supplemented with 5% sheep blood (Oxoid Limited, Hampshire, UK) with a *Staphylococcus pseudintermedius* streak [38]. The agar plates were incubated in a 5% CO_2-enriched atmosphere at 35 ± 2 °C for 48 h to detect the presence of other respiratory bacteria.

M. hyopneumoniae-specific antibody responses

Before primo-vaccination (D0), on the day of booster vaccination (D14), at challenge (D28), 2 weeks after challenge (D42) and at euthanasia (D56), serum samples were collected and the number of *M. hyopneumoniae* seropositive animals was determined with a commercial blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK) according to the manufacturer's instructions. Samples with optical density (OD) lower than 50% of the average OD of the buffer control were considered positive. Samples with OD-values equal or bigger than 50% of the average OD of the buffer control were considered negative.

Mycoplasma hyopneumoniae-specific IgG and IgA isotypes were measured in serum (diluted 1:200 and 1:100, respectively) with an in-house indirect ELISA using Tween 20-extracted *M. hyopneumoniae* antigens according to Matthijs et al. [21]. All samples were tested in duplicate. To relatively quantify the antibody levels, a standard curve was made using twofold serial dilutions of a positive reference serum corresponding to defined arbitrary units (1:800 and 1:200 dilution defined as 1 unit for IgG and IgA, respectively). Optical density values of the samples were interpolated from the standard curve using non-linear regression with least square fits in Graphpad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA).

Mycoplasma hyopneumoniae-specific IgA antibodies in BAL fluid collected 2 weeks after challenge infection (D42) and at euthanasia (D56) were measured with an in-house indirect ELISA as previously described [21]. The BAL fluid was tested undiluted and in duplicate. Antibody levels were also relatively quantified as described above using a standard curve made with positive BAL fluid (1:32 dilution defined as 1 unit). Animals with values higher than 0 arbitrary units were considered as positive, while animals with values equal to 0 arbitrary units were classified as negative.

T cell assays

Shortly before challenge infection (D28) and at euthanasia (D56), blood samples were collected to assess *M. hyopneumoniae*-specific T cell responses according to Matthijs et al. [21]. Peripheral blood mononuclear cells (PBMCs) were isolated and restimulated in vitro overnight (18 h) with *M. hyopneumoniae* F7.2C bacterin. For the last 4 h, Brefeldin A was added to inhibit cytokine release and allow intracellular detection of cytokines. Subsequently, cells were harvested and the percentage of cytokine-producing T cells was measured by flow cytometry (FCM) using a 5-step 6-colour staining protocol. Briefly, following incubation with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA), cells were incubated with anti-CD4 (clone 74-12-4, Southern Biotech, Birmingham, AL, USA) and anti-CD8β (clone PG164A, WSU, Pullman, WA, USA) antibodies and then with their corresponding secondary antibodies anti-mouse IgG2b AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) and anti-mouse IgG2a PE-Cy7 (Abcam, Cambridge, UK). After fixation and permeabilization of the cells with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution kit (Becton–Dickinson, Franklin Lakes, NJ, USA), intracellular cytokines were stained with directly coupled anti-human TNF-α AlexaFluor 647 (clone MAb11, BioLegend, San Diego, CA, USA), anti-pig IFN-γ PerCP-Cy5.5 (clone P2G10, Becton–Dickinson) and anti-human IL-17A PE (clone SCPL1362, Becton–Dickinson). All samples were acquired on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) and the analysis performed with the FlowJo™ software (Tree Star Inc., Ashland, OR, USA). For each animal, samples were restimulated in triplicate cultures and analysed

separately. To define whether an animal had *M. hyopneumoniae*-specific circulating cells, a threshold value was calculated as the mean % of cytokine-producing $cells_{all\ control\ animals} + 3*SD_{all\ control\ animals}$ for D28, and as the mean % of cytokine-producing $cells_{NCG} + 3*SD_{NCG}$ for D56. Animals with values above the threshold were identified as positive, while animals with values equal to or below the threshold were classified as negative.

Cytokines in BAL fluid

The BAL fluid collected at D42 and D56 was tested undiluted for the presence of IL-1β (Porcine IL-1 beta/IL-1F2 DuoSet® ELISA, R&D Systems, Minneapolis, MN, USA), IL-6 (Porcine IL-6 DuoSet® ELISA, R&D Systems, Minneapolis, MN, USA), IFN-γ (Swine IFN-γ Antibody Pair, Invitrogen™, ThermoFisher Scientific) and TNF-α (Swine TNF-α CytoSet™, Invitrogen™, ThermoFisher Scientific) using a sandwich ELISA according to the manufacturer's instructions. The OD-values were converted to cytokine levels by means of a standard curve.

Statistical analyses

The RDS data was averaged for the periods D-6 until D56, D-6 until D27 and D28 until D56, and analysed using a repeated measures ANOVA. Pairwise comparisons between groups were obtained with Scheffé's post hoc test. The T cell data was analysed using a one-way ANOVA with Tukey–Kramer's post hoc test for pairwise comparisons. The parameters ADG, macroscopic lung lesions, microscopic lung lesions, % air, log copies *M. hyopneumoniae* DNA in BAL fluid, *M. hyopneumoniae*-specific IgG, *M. hyopneumoniae*-specific IgA (serum, BAL fluid), IL-1, IL-6, IFN-γ and TNF-α were not normally distributed according to the Shapiro–Wilk's test, and were analysed using a Kruskal–Wallis ANOVA followed by Dunn's post hoc test. Adjusted *P* values were computed to account for multiple comparisons, except for Scheffé's post hoc test, which is already quite conservative [39]. The NCG was not included in the statistical analyses as this group only served as a sentinel group. Statistical analyses of efficacy parameters were conducted in SPSS 24 for Windows (IBM, Armonk, NY, USA). Immune response parameters were analysed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical results were considered significant when $P \leq 0.05$.

Results

Clinical and performance parameters

General health, severity of coughing (daily RDS) and ADG of each piglet were closely monitored during the entire study. One piglet from the PCG died during anaesthesia on D28. This piglet was excluded from the RDS and ADG analyses. On D42, one piglet from the Lipo_DDA:TDB group showed severe abdominal breathing after blood sampling. Therefore, BAL fluid was not collected from that animal on D42.

None of the non-challenge control animals coughed, except for one pig on D44 (score 2, mild coughing in rest). An increase in the mean RDS was first observed in group Lipo_DDA:TDB from 6 to 8 days post challenge (DPC) onwards, followed by groups SWE_TLR, PCG and PLGA_TLR, respectively from 8, 9 and 10 DPC onwards. After several days, coughing decreased in the vaccinated groups, while it continued at a high level in the PCG until the end of the study (Figure 1). After challenge infection (D28–56), formulation SWE_TLR induced the highest reduction in mean RDS compared to the PCG (61.90%), followed by formulations PLGA_TLR and Lipo_DDA:TDB (50.34% and 38.78%, respectively). However, the reduction of coughing was only statistically significant for group SWE_TLR ($P \leq 0.05$; Table 2).

For each group, the mean ADG from D0–56, D0–28 and D28–56 is shown in Table 2. After challenge infection (D28–56), ADG from group SWE_TLR was significantly higher compared to the other vaccinated groups ($P \leq 0.05$).

Macroscopic and microscopic lung lesions

At euthanasia (D56), lungs were collected and scored for macroscopic and microscopic lung lesions. The percentage of lung area occupied by air was measured using image analysis. The results are presented in Table 2. Macroscopic lung lesions were observed in all animals from the PCG, and in 7/12 animals from each of the vaccinated groups. In the NCG, none of the animals either had macroscopic lung lesions, nor microscopic lung lesion scores higher than 2. All vaccinated groups had a significantly lower macroscopic lesion score compared to the PCG ($P \leq 0.05$). Formulation SWE_TLR induced the highest reduction in macroscopic lung lesions compared to the PCG (88.38%), while formulations PLGA_TLR and Lipo_DDA:TDB reduced macroscopic lung lesions with 81.11% and 69.88%, respectively. All three formulations significantly reduced microscopic lung lesions ($P \leq 0.05$), and the highest reduction was again observed in group SWE_TLR. Groups Lipo_DDA:TDB and SWE_TLR had a significantly higher percentage of lung area occupied by air compared to the PCG ($P \leq 0.05$).

Quantitative PCR for *M. hyopneumoniae* DNA and routine bacteriological culture on bronchoalveolar lavage fluid

The number of animals positive for *M. hyopneumoniae* DNA in BAL fluid and mean log copies *M.*

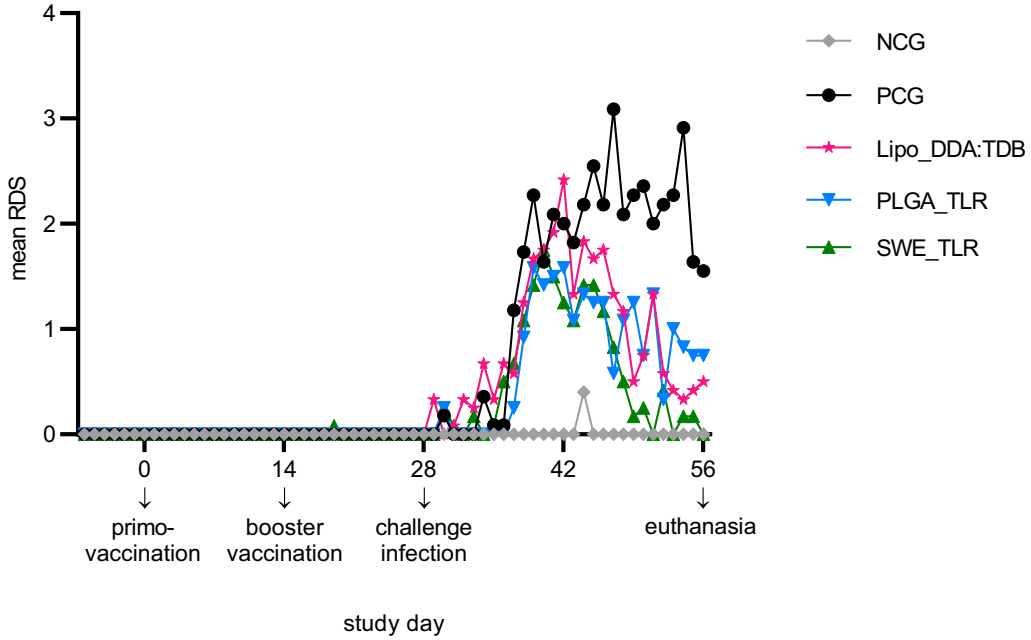

Figure 1 Mean respiratory disease score for each group during the entire study. NCG: non-challenge control group (PBS-injected, non-challenge infected), PCG: PBS-injected control group (PBS-injected, challenge infected), RDS: respiratory disease score.

Table 2 Overview of the efficacy data

Parameter	Study day	NCG	PCG	Lipo_DDA:TDB	PLGA_TLR	SWE_TLR	*P* value
RDS	−6 until 56	0.01 ± 0.01	0.68 ± 0.32^{b}	0.42 ± 0.33^{ab}	0.33 ± 0.35^{ab}	0.26 ± 0.15^{a}	0.011
	−6 until 27	0.00 ± 0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{a}	0.415
	28 until 56	0.01 ± 0.03	1.47 ± 0.69^{b}	0.90 ± 0.73^{ab}	0.73 ± 0.77^{ab}	0.56 ± 0.33^{a}	0.011
ADG in g/pig/d	0 until 56	691 ± 69	655 ± 109^{a}	610 ± 90^{a}	628 ± 97^{a}	688 ± 49^{a}	0.065
	0 until 28	620 ± 47	592 ± 104^{a}	560 ± 104^{a}	586 ± 106^{a}	581 ± 70^{a}	0.526
	28 until 56	762 ± 98	739 ± 141^{ab}	660 ± 96^{a}	669 ± 127^{a}	795 ± 69^{b}	0.004
Number of animals with macroscopic lung lesions (% positive animals)	56	0/5 (0.00)	11/11 (100.00)	7/12 (58.33)	7/12 (58.33)	7/12 (58.33)	–
Macroscopic lung lesion score	56	0.00 ± 0.00	7.57 ± 5.18^{b}	2.28 ± 3.55^{a}	1.43 ± 2.31^{a}	0.88 ± 1.01^{a}	0.002
Microscopic lung lesion score	56	1.13 ± 0.18	3.73 ± 0.94^{b}	2.11 ± 0.89^{a}	2.22 ± 0.87^{a}	1.78 ± 0.57^{a}	< 0.001
Percentage of lung area occupied by air	56	39.11 ± 6.26	23.38 ± 6.43^{b}	37.57 ± 5.99^{a}	34.82 ± 4.28^{ab}	42.15 ± 7.98^{a}	< 0.001
Number of animals positive for *M. hyopneumoniae* in BAL fluid (% positive animals)	42	0/5	11/11 (100.00)	9/11 (81.82)	9/12 (75.00)	6/12 (50.00)	–
	56	0/5	8/11 (72.73)	5/12 (41.67)	6/12 (50.00)	4/12 (33.33)	–
Log copies *M. hyopneumoniae* DNA/mL BAL fluid	42	0.00 ± 0.00	3.82 ± 0.71^{b}	2.50 ± 1.18^{ab}	2.20 ± 1.38^{a}	1.25 ± 1.28^{a}	< 0.001
	56	0.00 ± 0.00	1.74 ± 0.94^{a}	1.24 ± 1.34^{a}	1.76 ± 1.68^{a}	0.63 ± 0.74^{a}	0.082

Pigs were prime-boost vaccinated on D0 and D14 with three different experimental *M. hyopneumoniae* bacterins (Lipo_DDA:TDB, PLGA_TLR, SWE_TLR), challenge infected on D28–29 and euthanized on D56. For the parameters ADG, macroscopic lung lesion score, microscopic lung lesion score, % air and number of *M. hyopneumoniae* organisms in BAL fluid, a Kruskal–Wallis ANOVA was performed to determine statistical differences between the groups on each time point. For the parameter RDS, a repeated measurements ANOVA with Scheffé's post hoc test was performed. The NCG was not included in the statistical analyses. Groups that have no superscript in common are significantly different from each other ($P \leq 0.05$). All results are expressed as mean ± SD, unless otherwise stated.

NCG: non-challenge control group (non-vaccinated, non-challenge infected), PCG: PBS-injected control group (non-vaccinated, challenge infected), RDS: respiratory disease score, ADG: average daily gain, BAL: bronchoalveolar lavage.

hyopneumoniae DNA in BAL fluid are shown for each group in Table 2. Two weeks after challenge infection (D42), significantly lower numbers of *M. hyopneumoniae* organisms were detected in BAL fluid from groups PLGA_TLR and SWE_TLR compared to the PCG ($P \leq 0.05$). The reduction in log copies *M. hyopneumoniae*

DNA was 42.41% and 67.28%, respectively. Formulation Lipo_DDA:TDB reduced the number of *M. hyopneumoniae* organisms in BAL fluid with 34.55% compared to the PCG, but this reduction was not statistically significant ($P > 0.05$). At euthanasia (D56), all groups had a lower number of *M. hyopneumoniae* DNA in BAL fluid compared to D42 and no significant differences were observed between the groups ($P > 0.05$).

No other respiratory bacteria were detected after inoculating the BAL samples on Columbia blood agar plates.

M. hyopneumoniae-specific antibody responses

According to the commercial blocking ELISA (Additional file 1), all animals from group Lipo_DDA:TDB were positive for *M. hyopneumoniae*-specific antibodies in serum 2 weeks after booster vaccination (D28). In groups PLGA_TLR and SWE_TLR, 5/12 and 11/12 pigs seroconverted, respectively. Two weeks after challenge infection (D42), all pigs from the vaccinated groups were seropositive, together with 7/11 pigs from the PCG. At euthanasia (D56), all pigs from the vaccinated groups and PCG were seropositive. The pigs form the NCG remained serologically negative for *M. hyopneumoniae* during the entire study.

Mycoplasma hyopneumoniae-specific IgG and IgA levels in serum and *M. hyopneumoniae*-specific IgA levels in BAL fluid were quantified using an in-house indirect ELISA with positive reference serum or BAL fluid as a standard (Figures 2A–C). Two weeks after booster vaccination (D28), formulations Lipo_DDA:TDB and SWE_TLR induced a significant *M. hyopneumoniae*-specific IgG response ($P \leq 0.05$). Two weeks after challenge infection (D42) and at euthanasia (D56), all vaccinated groups had higher *M. hyopneumoniae*-specific IgG levels compared to the PCG. This was statistically significant for all vaccinated groups ($P \leq 0.05$) except group PLGA_TLR on D42 (Figure 2A).

Two weeks after primo-vaccination (D14) and 2 weeks after booster vaccination (D28), none of the vaccine formulations induced a significant *M. hyopneumoniae*-specific IgA response in serum ($P > 0.05$). Nevertheless, 2 weeks after challenge infection (D42), group Lipo_DDA:TDB showed a significantly higher *M. hyopneumoniae*-specific IgA response compared to the PCG and group PLGA_TLR ($P \leq 0.05$). Also, at euthanasia (D56), group Lipo_DDA:TDB had a significantly higher level of *M. hyopneumoniae*-specific IgA in serum compared to the PCG ($P \leq 0.05$; Figure 2B).

According to the in-house IgA ELISA on BAL fluid, respectively 9/11, 6/12 and 5/12 animals from groups Lipo_DDA:TDB, PLGA_TLR and SWE_TLR had *M. hyopneumoniae*-specific IgA antibodies in BAL fluid collected 2 weeks after challenge (D42). At euthanasia (D56), all animals from the vaccinated groups and the PCG were positive for *M. hyopneumoniae*-specific IgA. No IgA antibodies were detected in BAL fluid from the non-challenge control animals on both sampling days (Additional file 1). Two weeks after challenge infection (D42), group Lipo_DDA:TDB showed a significantly higher *M. hyopneumoniae*-specific IgA response in BAL fluid compared to the PCG ($P \leq 0.05$). At euthanasia (D56), groups Lipo_DDA:TDB and SWE_TLR had significantly higher *M. hyopneumoniae*-specific IgA levels in BAL fluid compared to the PCG, and group Lipo_DDA:TDB was also significantly higher than group PLGA_TLR ($P \leq 0.05$; Figure 2C).

T cell assays

The results of the *M. hyopneumoniae*-specific T cell responses detected in the blood 2 weeks after booster vaccination (D28) are presented in Figures 3A–C. Group SWE_TLR had a significantly higher percentage of TNF-producing $CD4^+$ (Th1) cells compared to the PCG and group PLGA_TLR ($P \leq 0.05$). However, in this group only five animals were above the threshold. In the Lipo_DDA:TDB group four animals and in group PLGA_TLR one animal were above the cut-off value (Figure 3A). For the $CD4^+$ IL-$17A^+$ (Th17) cells, one pig from group Lipo_DDA:TDB and two pigs from group SWE_TLR were above the cut-off (Figure 3B). Two pigs from group Lipo_DDA:TDB, 1 pig from group PLGA_TLR and three pigs from group SWE_TLR showed circulating $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells at the time of sampling. The percentage $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells in group SWE_TLR was significantly higher compared to the PLGA_TLR group ($P \leq 0.05$; Figure 3C).

Four weeks after challenge (D56), group PLGA_TLR had a significantly higher percentage $CD4^+$ $TNF^+IFN\text{-}\gamma^+$ (Th1) cells compared to the PCG and the other two vaccinated groups ($P \leq 0.05$), although merely five animals appeared to have such cells in the blood (Figure 3D). In the blood of one pig from the PCG and 1 pig of the Lipo_DDA:TDB group $CD4^+$ IL-$17A^+$ T cells were detected (Figure 3E). Only in the PLGA_TLR group one animal had $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells above the defined threshold (Figure 3F).

Cytokines in BAL fluid

The concentrations of IL-1β, IL-6, IFN-γ and TNF-α in BAL fluid collected on D42 and D56 are presented for each group in Figures 4A–D. Two weeks after challenge infection (D42), the IL-1β concentration in BAL fluid was significantly higher in group Lipo_DDA:TDB compared to the PCG and group SWE_TLR ($P \leq 0.05$). At euthanasia (D56), group PLGA_TLR had a significantly lower

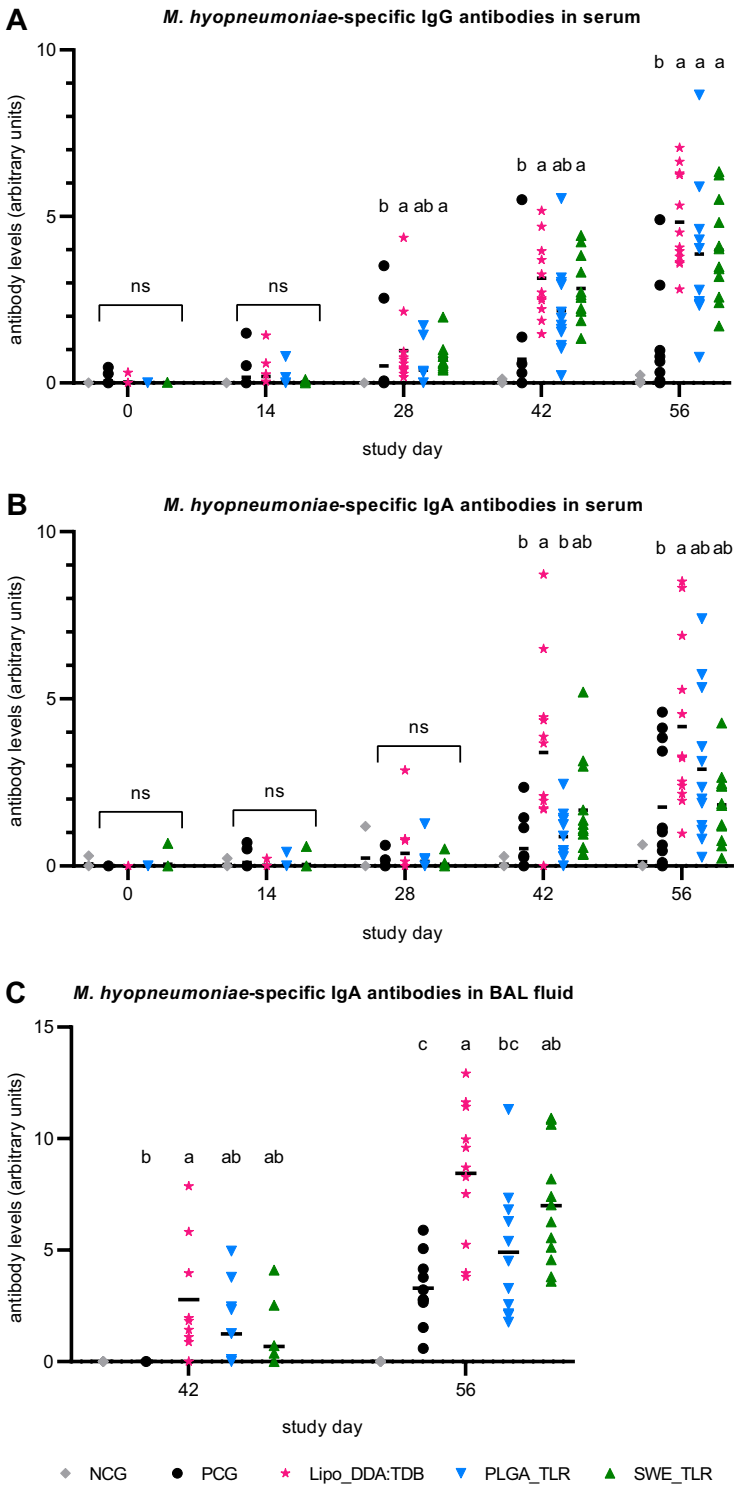

Figure 2 Antibody levels following vaccination and challenge infection. Pigs were prime-boost vaccinated on D0 and D14 with three different experimental *M. hyopneumoniae* bacterins (Lipo_DDA:TDB, PLGA_TLR, SWE_TLR), challenge infected on D28–29 and euthanized on D56. *M. hyopneumoniae*-specific IgG (**A**) and IgA (**B**) antibodies in serum and *M. hyopneumoniae*-specific IgA in BAL fluid (**C**) were determined by indirect ELISA. Individual animals are shown. For each time point, significance was calculated using a Kruskal–Wallis ANOVA. The NCG was not included in the statistical analyses. Groups that have no superscript in common are significantly different from each other ($P \leq 0.05$). NCG, non-challenge control group (PBS-injected, non-challenge infected); PCG, PBS-injected control group (PBS-injected, challenge infected).

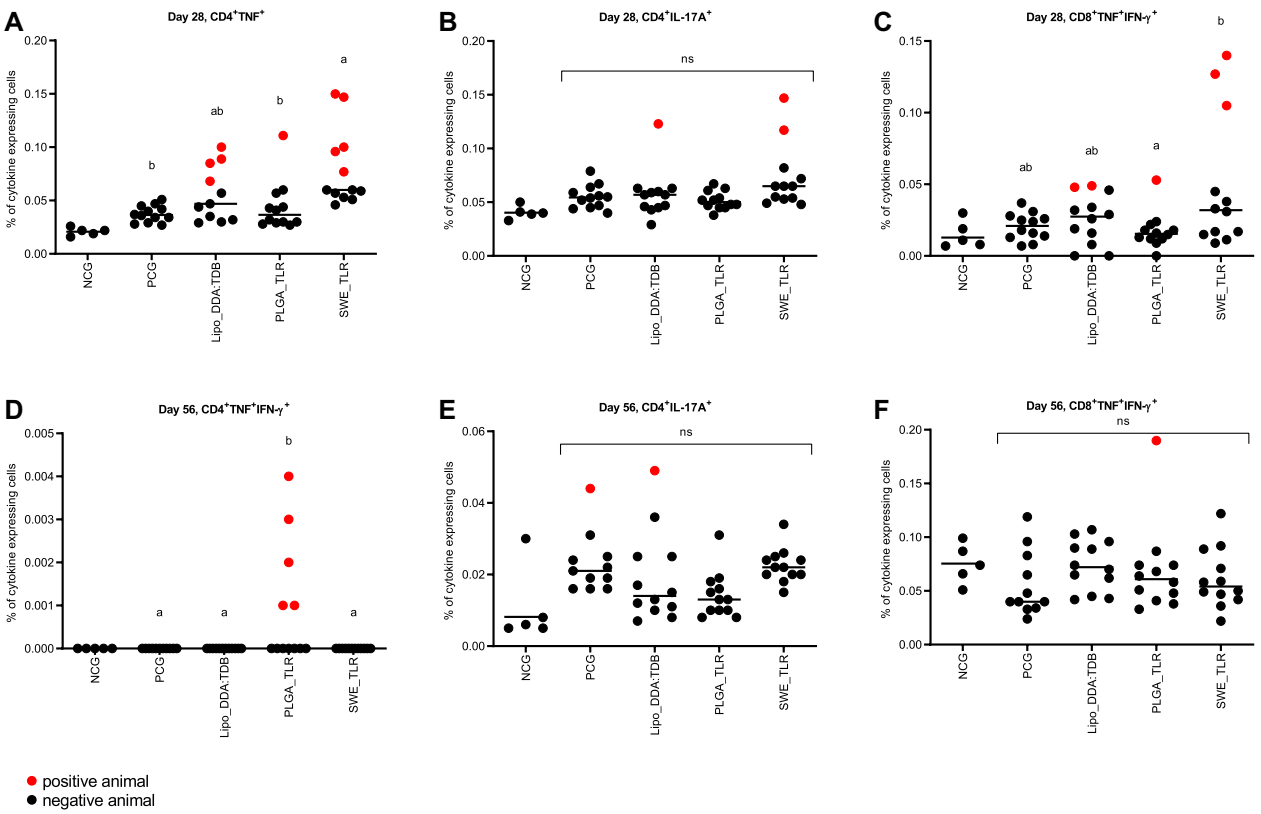

Figure 3 ***M. hyopneumoniae*-specific T cell responses following vaccination and challenge infection.** Pigs were prime-boost vaccinated on D0 and D14 with three different experimental *M. hyopneumoniae* bacterins (Lipo_DDA:TDB, PLGA_TLR, SWE_TLR), challenge infected on D28–29 and euthanized on D56. *M. hyopneumoniae*-specific T cells were determined by in vitro restimulation of PBMC followed by intracellular cytokine staining and multicolour flow cytometry. Following doublet exclusion, live cells were gated and the percentage of TNF^+ $CD4^+$, $IFN\gamma^+TNF^+$ double positive $CD4^+$ and $CD8\beta^+$ T cells, and IL-$17A^+$ $CD4^+$ T cells was determined. The mean values obtained from triplicate cultures for individual animals are shown. Positive animals are marked in red (defined as being above the mean + 3*SD of all control animals on D28 and being above the mean + 3*SD of the NCG on D56). The horizontal line in each group represents the group mean. For each time point, significance between groups was calculated using one-way ANOVA followed by Tukey–Kramer's test. The NCG was not included in the statistical analyses. Groups that have no superscript in common are significantly different from each other ($P \leq 0.05$). NCG, non-challenge control group (PBS-injected, non-challenge infected); PCG, PBS-injected control group (PBS-injected, challenge infected); PBMC, peripheral blood mononuclear cells.

IL-1β concentration compared to the PCG ($P \leq 0.05$; Figure 4A).

Only in BAL fluid from group Lipo_DDA:TDB a significant level of IFN-γ was detected on D42 ($P \leq 0.05$). At euthanasia (D56), group SWE-TLR had a significantly higher IFN-γ concentration compared to group PLGA_TLR ($P \leq 0.05$; Figure 4C).

Regarding IL-6 and TNF-α, no statistically significant differences were observed between the groups on both time points ($P > 0.05$; Figures 4B and D).

Discussion

The present study assessed the protective efficacy of three innovative *M. hyopneumoniae* bacterin formulations in a porcine experimental challenge model. The pigs were challenge infected with two *M. hyopneumoniae* field strains according to Michiels et al. [31]. These included strain F7.2C (the vaccine strain) and strain F1.12A, which were shown to differ from each other on a genomic [40], proteomic [23] and pathogenic [22] level. Challenge infection with two genetically different field strains might improve extrapolation to the field situation compared to experimental infection with only one strain, as the study from Michiels et al. demonstrated that most pigs were simultaneously infected with two or more genetically different *M. hyopneumoniae* strains under field conditions [41]. All animals from the PCG (PBS-injected, challenge infected) developed lung lesions, were positive for *M. hyopneumoniae* in BAL fluid and seroconverted, indicating that the challenge infection was successful. The values for the RDS and lung lesion scores in the PCG were comparable with those found in previous experimental studies using the same challenge model [31, 42].

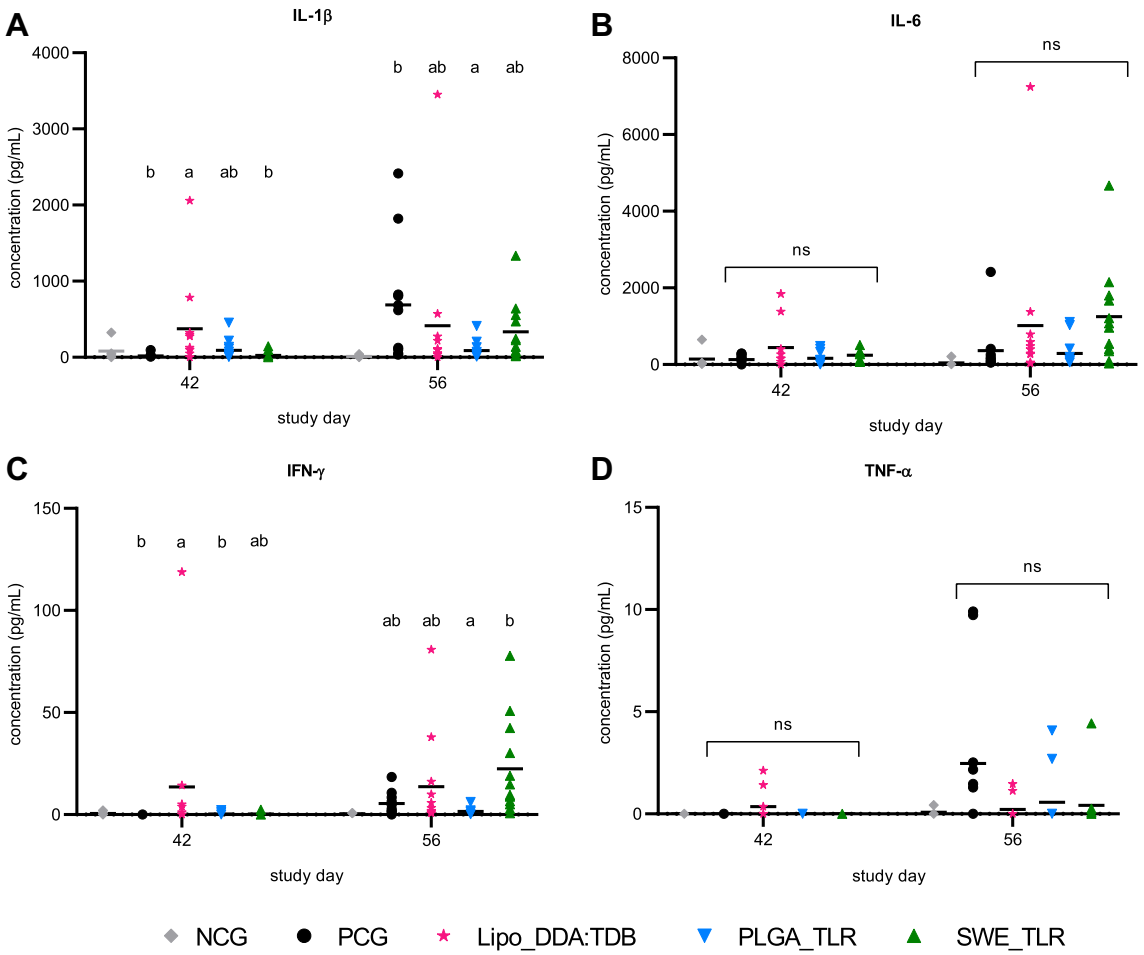

Figure 4 Cytokine concentrations (pg/mL) in bronchoalveolar lavage fluid. Pigs were prime-boost vaccinated on D0 and D14 with three different experimental *M. hyopneumoniae* bacterins (Lipo_DDA:TDB, PLGA_TLR, SWE_TLR), challenge infected on D28–29 and euthanized on D56. IL-1β (**A**), IL-6 (**B**), IFN-γ (**C**) and TNF-α (**D**) concentrations in BAL fluid were measured using commercial sandwich ELISAs. For each time point, significance was calculated using a Kruskal–Wallis ANOVA. The NCG was not included in the statistical analyses. Groups that have no superscript in common are significantly different from each other ($P \leq 0.05$). NCG, non-challenge control group (PBS-injected, non-challenge infected), PCG, PBS-injected control group (PBS-injected, challenge infected), BAL, bronchoalveolar lavage.

According to the in-house serum ELISAs, some animals appeared to have *M. hyopneumoniae*-specific antibodies before vaccination and/or challenge infection (animals with values higher than 0 arbitrary units; Figure 2). These animals most likely tested false positive due to non-specific binding, since the study animals were obtained from a *M. hyopneumoniae*-free farm. Moreover, the particular pigs tested negative in the commercial blocking ELISA on those time points. While the commercial kit uses a highly specific monoclonal antibody against a conserved epitope of the *M. hyopneumoniae* 74 kDa protein, our in-house indirect ELISA is based on Tween 20-extracted proteins of *M. hyopneumoniae*. Such crude antigen preparations more easily allow cross-reactions with antibodies against the closely related *M. flocculare*, a commonly occurring commensal in the respiratory tract of pigs [43]. However, an additional *M. flocculare* ELISA to confirm the presence of such antibodies was not performed. In consequence, it remains uncertain whether all obtained in-house serum ELISA results are purely due to a *M. hyopneumoniae* response.

All three vaccine formulations were able to reduce clinical signs, macroscopic lung lesions and histopathological lung lesions, with formulation SWE_TLR being the most effective (RDS −61.9%, macroscopic lung lesions −88.4%, log copies *M. hyopneumoniae* DNA in BAL fluid −67.3%). The improvements obtained with formulation SWE_TLR seemed similar to or sometimes even better (adding up to 40% and 58% to the reduction of macroscopic lung lesions and log copies *M. hyopneumoniae*

DNA, respectively) than the results obtained with commercial *M. hyopneumoniae* bacterins under experimental conditions [11, 31, 44, 45]. However, due to differences in the experimental settings (i.e. age of vaccination, challenge strains, one-shot vs. two-shot vaccination), comparisons with other trials remain speculative. In order to properly compare the protective efficacy of the experimental vaccines with the protection levels induced by commercial vaccines, a commercial two-shot vaccine should have been included in the experimental design. Interestingly, the protective efficacy of the highly virulent F7.2C strain formulated as a bacterin in combination with an aqueous adjuvant was assessed in a previous study [11]. However, in that study, the experimental vaccine formulation did not offer significant protection against experimental infection. This might be explained by the use of a less potent adjuvant and/or the lower antigen load (7.7 log10 CCU/mL) of the vaccine. Next to that, it should be mentioned that the vaccination was partially homologous to the challenge infection in this study, as strain F7.2C was used to construct the vaccines and was also one of the two challenge strains. One might suggest that this could result in better protection compared to challenge with strains different from the vaccine. Nevertheless, Villarreal et al. showed that vaccination with a bacterin homologous to the strain used for challenge infection did not result in an increased protection when compared to bacterins containing genetically heterologous strains [11].

Two weeks after challenge infection, groups SWE_TLR and PLGA_TLR had significantly lower numbers of *M. hyopneumoniae* organisms in BAL fluid compared to the PCG, indicating a lower shedding of *M. hyopneumoniae* in vaccinated pigs. However, like the current commercial vaccines, the experimental vaccine formulations from this study could not prevent colonization of the pathogen in the respiratory tract of the pigs.

Group SWE_TLR was the only group that had a higher ADG from the day of challenge until euthanasia. However, these findings were not statistically significant, most likely due to the small number of animals included in the study, the high SD of this parameter and the rather short study period [11, 31]. Further research including more animals and raised under field conditions is needed to obtain more reliable data on the impact of these experimental vaccines on performance parameters such as ADG and feed conversion ratio (FCR).

In accordance with the results from a previous study [21], formulation Lipo_DDA:TDB was the most potent in inducing a serological IgG response. However, the reduction in lung lesions was the lowest in group Lipo_DDA:TDB, confirming once more that systemic antibodies do not correlate with protection against EP [12]. Mucosal IgA is considered important to control *M. hyopneumoniae* infection, as adherence of the bacteria to the cilia of respiratory epithelium is the first step in the pathogenesis [13]. In the study from Matthijs et al. [21], only one animal from group SWE_TLR had *M. hyopneumoniae*-specific IgA antibodies in BAL fluid 2 weeks after booster vaccination. In the present study, groups Lipo_DDA:TDB and SWE_TLR had significantly more *M. hyopneumoniae*-specific IgA in BAL fluid collected 4 weeks after challenge compared to the non-vaccinated animals. Similar observations were made in previous studies [12, 45]. This increase of specific IgA in BAL fluid from the vaccinated groups indicates an anamnestic immune response, and suggests that priming of the mucosal immune system is possible after parenteral vaccine administration. As T cells are required for isotype switching, it also confirms the priming of specific T helper cells by all three vaccines. Although the *M. hyopneumoniae*-specific IgA levels in BAL fluid are not completely in line with its levels in serum, it can however not be stated with certainty that all the detected antigen-specific IgA in BAL fluid is produced locally. As with the *M. hyopneumoniae*-specific serum antibodies, the formulation inducing the highest antigen-specific IgA levels after challenge infection did not offer the highest protection. This indicates that also other arms of the immune system play an important role in the protection against EP. It is also possible that mucosal IgA is only protective if induced pre-challenge. Clearly, developing an effective mucosal vaccine is required to address this question.

Two weeks after booster vaccination (D28), a significantly higher percentage of *M. hyopneumoniae*-specific Th1 cells was observed in group SWE_TLR. Some animals in group Lipo_DDA:TDB also appeared to have such cells in the blood circulation. These results are in accordance with the results from the study of Matthijs et al. [21], where formulations Lipo_DDA:TDB and SWE_TLR induced a stronger *M. hyopneumoniae*-specific circulating Th1 response 2 weeks after booster vaccination. Only a few vaccinated animals had *M. hyopneumoniae*-specific circulating Th17 and $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells, while the study of Matthijs et al. [21] showed a significant Th17 response in group PLGA_TLR and moderate to strong $CD8^+$ T cell responses in groups SWE_TLR and Lipo_DDA:TDB, respectively. However, this lack of detectable *M. hyopneumoniae*-specific T cells should not be interpreted as a lack of T cell priming, as the frequency of antigen-specific T cells circulating in the peripheral blood compartment is a very dynamic process and changes over time. Following the expansion of specific T cells observed during a recall response (booster vaccination or challenge for this experiment), the contraction phase corresponds to a huge decrease in the frequency

of antigen-specific T cells before they become memory cells. Even though memory cells are still circulating in the peripheral blood, their frequency is low as most memory cells recirculate between lymphoid tissue and blood, migrate to peripheral sites or the bone marrow for long-term survival [46, 47]. It appears that in this study formulation Lipo_DDA:TDB induced less circulating specific T cells compared to the study of Matthijs et al. [21], which might also be due to the fact that this vaccine was only applied IM in this study, while it was applied intradermally and IM at primo-vaccination in the previous study. The change in administration route was necessary due to severe local reactions at the intradermal injection site [21]. Interestingly, the SWE_TLR formulation appeared the most capable of inducing cellular immunity detectable in the blood, and also offered the highest protection. Altogether, the data of this study support the hypothesis that cellular immunity is important for protection against EP.

Two weeks after challenge infection, group Lipo_DDA:TDB had very high IL-1β levels compared to the other groups. This group also had the highest RDS at that time point. Several studies have associated the excessive production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α with the development of *M. hyopneumoniae*-induced pneumonia [48–50]. According to Marchioro et al. [36], vaccination might reduce lung damage by regulating the release of these pro-inflammatory cytokines. However, in this study vaccination did not strongly impact the concentration of pro-inflammatory cytokines in the BAL fluid.

In conclusion, all formulations were able to reduce clinical symptoms, macro- and microscopic lung lesions and the *M. hyopneumoniae* DNA load in the lung, with the oil-in-water formulation delivering a cocktail of TLR-ligands being the most effective. As the number of animals is limited in experimental infection studies, further research including more animals and raised under field conditions is needed to confirm the present results, and especially to assess the effects of the different vaccine formulations from this study on the reduction of performance losses (ADG, FCR) due to *M. hyopneumoniae* infections.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-019-0709-0.

Additional file 1. Results of the *M. hyopneumoniae*-specific antibodies measured at different time points in serum and in BAL fluid. Pigs were prime-boost vaccinated on D0 and D14 with three different experimental *M. hyopneumoniae* bacterins (Lipo_DDA:TDB, PLGA_TLR, SWE_TLR), challenge infected on D28–29 and euthanized on D56. *M. hyopneumoniae*-specific antibodies were determined by the IDEIA™ *Mycoplasma hyopneumoniae* EIA kit (Oxoid Limited, Hampshire, UK) and by indirect in-house ELISAs. For the in-house ELISAs, NetOD-values were calculated by subtracting the OD-value of the blank from the OD-value of the sample. BAL, bronchoalveolar lavage; NCG, non-challenge control group (PBS-injected, non-challenge infected); PCG, PBS-injected control group (PBS-injected, challenge infected); OD, optical density.

Abbreviations

ADG: average daily gain; BAL: bronchoalveolar lavage; CCU: colour changing units; c-di-AMP: cyclic diadenylate monophosphate; CpG: CpG oligodeoxynucleotides SL03; D: day; DDA: dimethyl dioctadecylammonium; EP: enzootic pneumonia; FCM: flow cytometry; FCR: feed conversion ratio; Ig: immunoglobulin; IM: intramuscular, intramuscularly; NCG: non-challenge control group; OD: optical density; PAM: Pam3Cys-SK4; PBS: phosphate buffered saline; PCG: PBS-injected group; PLGA: poly(lactic-*co*-glycolic acid); SD: standard deviation; SWE: squalene-in-water emulsion; TDB: trehalose 6,6-dibehenate; Th: T helper; TLR: Toll-like receptor.

Acknowledgements

The authors would like to thank Marleen Foubert for her help with the ELISAs.

Authors' contributions

AMFM, GA, ASc, OGN, GTB, BD, AM performed the animal experimentation, acquisition and analyses of data. VJ, CBQ, NC designed and produced the vaccines. DM, CBQ, FB, FH and ASu designed and supervised the overall project. All authors read and approved the final manuscript.

Funding

This study was financially supported by the European H2020 Project SAPHIR (Project No. 633184).

Ethics approval and consent to participate

The study protocol was approved by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (Approval Number EC2016/91).

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium. [2] Institute of Virology and Immunology, Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland. [3] Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Länggassstrasse 120, 3012 Bern, Switzerland. [4] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium. [5] Vaccine Formulation Laboratory, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland. [6] Laboratory of Veterinary Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium.

Received: 14 June 2019 Accepted: 25 September 2019
Published online: 08 November 2019

References

1. Maes D, Segales J, Meyns T, Sibila M, Pieters M, Haesebrouck F (2008) Control of *Mycoplasma hyopneumoniae* infections in pigs. Vet Microbiol 126:297–309
2. Holst S, Yeske P, Pieters M (2015) Elimination of *Mycoplasma hyopneumoniae* from breed-to-wean farms: a review of current protocols with emphasis on herd closure and medication. J Swine Health Prod 23:321–330
3. Goodwin R, Whittlestone P (1963) Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. Br J Exp Pathol 44:291–299

4. Maes D, Sibila M, Kuhnert P, Segalés J, Haesebrouck F, Pieters M (2018) Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. Transbound Emerg Dis 65:110–124
5. Jensen C, Ersbøll AK, Nielsen JP (2002) A meta-analysis comparing the effect of vaccines against *Mycoplasma hyopneumoniae* on daily weight gain in pigs. Prev Vet Med 54:265–278
6. Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, Verbeke W, Viaene J, de Kruif A (1999) Effect of vaccination against *Mycoplasma hyopneumoniae* in pig herds with an all-in/all-out production system. Vaccine 17:1024–1034
7. Thacker EL, Thacker BJ, Boettcher TB, Jayappa H (1998) Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. J Swine Health Prod 6:107–112
8. Meyns T, Dewulf J, De Kruif A, Calus D, Haesebrouck F, Maes D (2006) Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. Vaccine 24:7081–7086
9. Pieters M, Fano E, Pijoan C, Dee S (2010) An experimental model to evaluate *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated sentinel pigs. Can J Vet Res 74:157–160
10. Villarreal I, Meyns T, Dewulf J, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D (2011) The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. Vet J 188:48–52
11. Villarreal I, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D (2012) Effect of challenge of pigs previously immunised with inactivated vaccines containing homologous and heterologous *Mycoplasma hyopneumoniae* strains. BMC Vet Res 8:2
12. Djordjevic S, Eamens G, Romalis L, Nicholls P, Taylor V, Chin J (1997) Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Aust Vet J 75:504–511
13. Thacker EL, Thacker BJ, Kuhn M, Hawkins PA, Waters WR (2000) Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am J Vet Res 61:1384–1389
14. Dobbs NA, Odeh AN, Sun X, Simecka JW (2009) The multifaceted role of T cell-mediated immunity in pathogenesis and resistance to mycoplasma respiratory disease. Curr Trends Immunol 10:1–19
15. Seo HW, Han K, Oh Y, Park C, Choo EJ, Kim S-H, Lee B-H, Chae C (2013) Comparison of cell-mediated immunity induced by three commercial single-dose *Mycoplasma hyopneumoniae* bacterins in pigs. J Vet Med Sci 75:245–247
16. Martelli P, Saleri R, Cavalli V, De Angelis E, Ferrari L, Benetti M, Ferrarini G, Merialdi G, Borghetti P (2014) Systemic and local immune response in pigs intradermally and intramuscularly injected with inactivated *Mycoplasma hyopneumoniae* vaccines. Vet Microbiol 168:357–364
17. Jaffar Z, Ferrini ME, Herritt LA, Roberts K (2009) Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. J Immunol 182:4507–4511
18. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, Goldman SJ, Dunussi-Joannopoulos K, Williams CM, Wright JF, Fouser LA (2007) An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol 179:7791–7799
19. Jones HP, Tabor L, Sun X, Woolard MD, Simecka JW (2002) Depletion of CD8+T cells exacerbates CD4+Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease. J Immunol 168:3493–3501
20. Simionatto S, Marchioro SB, Maes D, Dellagostin OA (2013) *Mycoplasma hyopneumoniae*: from disease to vaccine development. Vet Microbiol 165:234–242
21. Matthijs AMF, Auray G, Jakob V, García-Nicolás O, Braun RO, Keller I, Bruggmann R, Devriendt B, Boyen F, Guzmán CA, Michiels A, Haesebrouck F, Nicolas C, Barnier-Quer C, Maes D, Summerfield A (2019) System immunology characterization of novel vaccine formulations for *Mycoplasma hyopneumoniae* bacterins. Front Immunol 10:1087
22. Vicca J, Stakenborg T, Maes D, Butaye P, Peeters J, de Kruif A, Haesebrouck F (2003) Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet Microbiol 97:177–190
23. Calus D, Baele M, Meyns T, de Kruif A, Butaye P, Decostere A, Haesebrouck F, Maes D (2007) Protein variability among *Mycoplasma hyopneumoniae* isolates. Vet Microbiol 120:284–291
24. Auray G, Keller I, Python S, Gerber M, Bruggmann R, Ruggli N, Summerfield A (2016) Characterization and transcriptomic analysis of porcine blood conventional and plasmacytoid dendritic cells reveals striking species-specific differences. J Immunol 197:4791–4806
25. Vreman S, Auray G, Savelkoul HF, Rebel A, Summerfield A, Stockhofe-Zurwieden N (2018) Neonatal porcine blood derived dendritic cell subsets show activation after TLR2 or TLR9 stimulation. Dev Comp Immunol 84:361–370
26. Braun RO, Python S, Summerfield A (2017) Porcine B cell subset responses to toll-like receptor ligands. Front Immunol 8:1044
27. Christensen D, Foged C, Rosenkrands I, Nielsen HM, Andersen P, Agger EM (2007) Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. Biochim Biophys Acta 1768:2120–2129
28. Singh M, Briones M, Ott G, O'Hagan D (2000) Cationic microparticles: a potent delivery system for DNA vaccines. Proc Natl Acad Sci USA 97:811–816
29. Ventura R, Brunner L, Heriyanto B, de Boer O, O'Hara M, Huynh C, Suhardono M, Collin N (2013) Technology transfer of an oil-in-water vaccine-adjuvant for strengthening pandemic influenza preparedness in Indonesia. Vaccine 31:1641–1645
30. Friis N (1975) Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* a survey. Nord Vete Med 27:337–339
31. Michiels A, Arsenakis I, Boyen F, Krejci R, Haesebrouck F, Maes D (2017) Efficacy of one dose vaccination against experimental infection with two *Mycoplasma hyopneumoniae* strains. BMC Vet Res 13:274
32. Halbur PG, Paul PS, Meng X-J, Lum MA, Andrews JJ, Rathje JA (1996) Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J Vet Diagn Invest 8:11–20
33. Sacristán RDP, Sierens A, Marchioro S, Vangroenweghe F, Jourquin J, Labarque G, Haesebrouck F, Maes D (2014) Efficacy of early *Mycoplasma hyopneumoniae* vaccination against mixed respiratory disease in older fattening pigs. Vet Rec 174:197
34. Hannan P, Bhogal B, Fish J (1982) Tylosin tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses. Res Vet Sci 33:76–88
35. Morris CR, Gardner IA, Hietala SK, Carpenter TE, Anderson RJ, Parker KM (1995) Seroepidemiologic study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Prev Vet Med 21:323–337
36. Marchioro SB, Maes D, Flahou B, Pasmans F, Sacristán RDP, Vranckx K, Melkebeek V, Cox E, Wuyts N, Haesebrouck F (2013) Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine. Vaccine 31:1305–1311
37. Marois C, Dory D, Fablet C, Madec F, Kobisch M (2010) Development of a quantitative real-time TaqMan PCR assay for determination of the minimal dose of *Mycoplasma hyopneumoniae* strain 116 required to induce pneumonia in SPF pigs. J Appl Microbiol 108:1523–1533
38. Villarreal I, Maes D, Vranckx K, Calus D, Pasmans F, Haesebrouck F (2011) Effect of vaccination of pigs against experimental infection with high and low virulence *Mycoplasma hyopneumoniae* strains. Vaccine 29:1731–1735
39. Maxwell SE, Delaney HD, Kelley K (2017) Designing experiments and analyzing data: a model comparison perspective, 3rd edn. Routledge, New York
40. Vranckx K, Maes D, Calus D, Villarreal I, Pasmans F, Haesebrouck F (2011) Multiple-locus variable-number tandem-repeat analysis is a suitable tool for differentiation of *Mycoplasma hyopneumoniae* strains without cultivation. J Clin Microbiol 49:2020–2023
41. Michiels A, Vranckx K, Piepers S, Sacristán RDP, Arsenakis I, Boyen F, Haesebrouck F, Maes D (2017) Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. Vet Res 48:2
42. Michiels A, Arsenakis I, Matthijs A, Boyen F, Haesaert G, Audenaert K, Eeckhout M, Croubels S, Haesebrouck F, Maes D (2018) Clinical impact of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol on the severity of an experimental *Mycoplasma hyopneumoniae* infection in pigs. BMC Vet Res 14:190

43. Feld NC, Qvist P, Ahrens P, Friis NF, Meyling A (1992) A monoclonal blocking ELISA detecting serum antibodies to *Mycoplasma hyopneumoniae*. Vet Microbiol 30:35–46
44. Arsenakis I, Panzavolta L, Michiels A, Sacristán RDP, Boyen F, Haesebrouck F, Maes D (2016) Efficacy of *Mycoplasma hyopneumoniae* vaccination before and at weaning against experimental challenge infection in pigs. BMC Vet Res 12:63
45. Strait EL, Rapp-Gabrielson VJ, Erickson BZ, Evans RB, Taylor LP, Yonkers TK, Keich RL, Jolie R, Thacker EL (2008) Efficacy of a *Mycoplasma hyopneumoniae* bacterin in pigs challenged with two contemporary pathogenic isolates of *M hyopneumoniae*. J Swine Health Prod 16:200–206
46. Woodland DL, Kohlmeier JE (2009) Migration, maintenance and recall of memory T cells in peripheral tissues. Nat Rev Immunol 9:153–161
47. Di Rosa F, Gebhardt T (2016) Bone marrow T cells and the integrated functions of recirculating and tissue-resident memory T cells. Front Immunol 7:51
48. Asai T, Okada M, Ono M, Mori Y, Yokomizo Y, Sato S (1994) Detection of interleukin-6 and prostaglandin E2 in bronchoalveolar lavage fluids of pigs experimentally infected with *Mycoplasma hyopneumoniae*. Vet Immunol Immunopathol 44:97–102
49. Ahn KK, Kwon D, Jung K, Ha Y, Seo MJ, Kim S-H, Kim M-Y, Cho K-D, Lee B-H, Chae C (2009) Identification of interleukin-1, tumor necrosis factor-α, and interleukin-6 expression in lungs from pigs naturally infected with *Mycoplasma hyopneumoniae* by in situ hybridization. J Vet Med Sci 71:441–445
50. Rodrıguez F, Ramırez G, Sarradell J, Andrada M, Lorenzo H (2004) Immunohistochemical labelling of cytokines in lung lesions of pigs naturally infected with *Mycoplasma hyopneumoniae*. J Comp Pathol 130:306–312

Publisher's Note

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 May 2019
doi: 10.3389/fimmu.2019.01087

Systems Immunology Characterization of Novel Vaccine Formulations for *Mycoplasma hyopneumoniae* Bacterins

Anneleen M. F. Matthijs[1], Gaël Auray[2,3], Virginie Jakob[4], Obdulio García-Nicolás[2,3], Roman O. Braun[2,3], Irene Keller[5,6], Rémy Bruggman[5], Bert Devriendt[7], Filip Boyen[8], Carlos A. Guzman[9], Annelies Michiels[1], Freddy Haesebrouck[8], Nicolas Collin[4], Christophe Barnier-Quer[4], Dominiek Maes[1†] and Artur Summerfield[2,3†]*

[1] Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [2] Institute of Virology and Immunology, Mittelhäusern, Switzerland, [3] Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland, [4] Vaccine Formulation Laboratory, University of Lausanne, Epalinges, Switzerland, [5] Interfaculty Bioinformatics Unit, Swiss Institute of Bioinformatics, University of Bern, Bern, Switzerland, [6] Department of Biomedical Research, University of Bern, Bern, Switzerland, [7] Laboratory of Veterinary Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [8] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [9] Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Brunswick, Germany

OPEN ACCESS

Edited by:
Cynthia Calzas, Institut National de la Recherche Agronomique (INRA), France

Reviewed by:
Max Bastian, Friedrich Loeffler Institut, Germany
Renukaradhya J. Gourapura, The Ohio State University, United States

***Correspondence:**
Artur Summerfield
artur.summerfield@vetsuisse.unibe.ch

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *08 January 2019*
Accepted: *29 April 2019*
Published: *24 May 2019*

Citation:
Matthijs AMF, Auray G, Jakob V, García-Nicolás O, Braun RO, Keller I, Bruggman R, Devriendt B, Boyen F, Guzman CA, Michiels A, Haesebrouck F, Collin N, Barnier-Quer C, Maes D and Summerfield A (2019) Systems Immunology Characterization of Novel Vaccine Formulations for Mycoplasma hyopneumoniae Bacterins. Front. Immunol. 10:1087. doi: 10.3389/fimmu.2019.01087

We characterized five different vaccine candidates and a commercial vaccine in terms of safety, immunogenicity and using a systems vaccinology approach, with the aim to select novel vaccine candidates against *Mycoplasma hyopneumoniae*. Seven groups of six *M. hyopneumoniae*-free piglets were primo- and booster vaccinated with the different experimental bacterin formulations, the commercial vaccine Hyogen® as a positive control or PBS as a negative control. The experimental bacterin was formulated with cationic liposomes + c-di-AMP (Lipo_AMP), cationic liposomes + Toll-like receptor (TLR) 2/1, TLR7, and TLR9 ligands (TLR ligands; Lipo_TLR), micro-particles + TLR ligands (PLGA_TLR), squalene-in-water emulsion + TLR ligands (SWE_TLR), or DDA:TDB liposomes (Lipo_DDA:TDB). Lipo_DDA:TDB and Lipo_AMP were the most potent in terms of serum antibody induction, and Lipo_DDA:TDB, Lipo_AMP, and SWE_TLR significantly induced Th1 cytokine-secreting T-cells. Only PLGA_TLR appeared to induce Th17 cells, but was unable to induce serum antibodies. The transcriptomic analyses demonstrated that the induction of inflammatory and myeloid cell blood transcriptional modules (BTM) in the first 24 h after vaccination correlated well with serum antibodies, while negative correlations with the same modules were found 7 days post-vaccination. Furthermore, many cell cycle and T-cell BTM upregulated at day seven correlated positively with adaptive immune responses. When comparing the delivery of the identical TLR ligands with the three formulations, we found SWE_TLR to be more potent in the induction of an early innate immune response, while the liposomal formulation more strongly promoted late cell cycle and T-cell BTM. For the PLGA formulation we found signs of a delayed and weak perturbation of these BTM. Lipo_AMP was found to be the most potent vaccine at inducing a BTM profile similar to that correlating with

adaptive immune response in this and other studies. Taken together, we identified four promising vaccine candidates able to induce *M. hyopneumoniae*-specific antibody and T-cell responses. In addition, we have adapted a systems vaccinology approach developed for human to pigs and demonstrated its capacity in identifying early immune signatures in the blood relating to adaptive immune responses. This approach represents an important step in a more rational design of efficacious vaccines for pigs.

Keywords: *Mycoplasma hyopneumoniae*, bacterins, safety, immune responses, transcriptomics

INTRODUCTION

Mycoplasma hyopneumoniae (M. hyopneumoniae) is the primary cause of enzootic pneumonia (EP), a chronic respiratory disease in pigs. The disease causes severe economic losses in swine-producing countries worldwide due to a reduced average daily weight gain of the pigs, a higher feed conversion ratio and an increased use of antimicrobial agents (1–3). Control of the disease can be achieved by optimizing management and housing conditions combined with medication and vaccination (2).

Vaccination with inactivated, adjuvanted whole-cell bacterins is practiced worldwide to control EP (4). However, current commercial vaccines only offer partial protection, have a limited effect on the transmission of the microorganism and cannot prevent colonization (5–7). Most commercial bacterins are based on the J-strain, a low virulent *M. hyopneumoniae* strain isolated in the UK in the sixties (8–10), and contain adjuvants including aluminum hydroxide, carbopol, mineral oil or biodegradable oil (4). The main effects of vaccination are a reduction in clinical symptoms, lung lesions, medication use, and performance losses (11, 12). Those effects may vary between pig herds (2), which could be partially explained by antigenic and pathogenic differences between the strains circulating in the herd and the vaccine strain (10).

The immune mechanisms leading to protection against *M. hyopneumoniae* infection are complex and not yet fully elucidated. *M. hyopneumoniae*-specific serum antibody concentrations induced by vaccination are not correlated with the severity of lung lesions in *M. hyopneumoniae*-infected pigs (5, 13), indicating that systemic antibodies play only a minor role in protective immunity. However, local mucosal antibodies (IgA) are considered important to prevent and control *M. hyopneumoniae*-induced pneumonia, as the adherence of the microorganism to the ciliated epithelium of the respiratory tract is the first step in the pathogenesis (14). Also, several studies suggest that systemic cell-mediated immune responses play a major role in disease protection (14–17).

Based on this knowledge, innovative bacterin formulations that include virulent *M. hyopneumoniae* strains formulated with adjuvants specifically designed to promote cellular immune responses could improve vaccine efficacy. Therefore, we developed three different vaccine formulations to deliver a cocktail of TLR 2/1, TLR 7, and TLR 9 ligands previously shown to potently activate porcine antigen presenting cells including dendritic cells (DC), monocytes and B cells (18, 19). The formulations included a liposomal, a micro-particle and an oil-in-water formulation. In addition, we developed a liposomal formulation to deliver a cyclic di-nucleotide targeting the STING pathway (20) as an alternative immunostimulant, and another cationic liposomal formulation to deliver a Mincle ligand, also previously found to be efficacious (21). All formulations were based on the *M. hyopneumoniae* strain F7.2C, a highly virulent field strain isolated in Belgium in 2000 (22, 23), and shown to be antigenically different from the J-strain (23).

Overall, the aim of this study was to assess the safety of these five novel bacterin formulations and characterize the immune responses induced by the formulations, compared to a commercial vaccine in order to select new promising vaccine candidates. To this end, *M. hyopneumoniae*-specific T cell responses and antibody responses were measured in pigs. For T cells, we focussed on Th1 and Th17 based on their known role in protective immunity against *Mycoplasma* infection, as identified in mouse models (24). Next to that, we employed a systems immunology approach to understand how different formulations modulate the immune system toward potent immunogenicity. This analysis employed "blood transcriptional modules" (BTM) defined for peripheral blood cells in human (25), which were adapted to pigs. This technique sheds light into the black box of the immune response by identifying pathways and networks of genes related to adaptive immune responses as previously demonstrated for human and sheep (25–34). Also, this approach has been shown to possess more discriminative power for analyses of peripheral blood leukocytes during vaccination when compared to gene sets based on canonical pathways (25). Our work has demonstrated the possibilities of such novel approaches in vaccinology and identified vaccine candidates for further exploration.

Abbreviations: EP, enzootic pneumonia; CCU, color changing units; PLGA, poly(lactic-co-glycolic acid); SWE, squalene-in-water emulsion; DDA, dimethyl dioctadecylammonium; DPPC:DC-Chol, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and dimethylaminoethane carbamoyl cholesterol; TDB, trehalose 6,6-dibehenate; c-di-AMP, cyclic diadenylate monophosphate; PAM, Pam3Cys-SK4; CpG, CpG ODN SL03; PS, particle size; Pdi, polydispersity index; ZP, zeta potential; IM, intramuscularly; ID, intradermally; ISR, injection site reaction; ADG, average daily gain; BAL, bronchoalveolar lavage; GSEA, gene set enrichment analysis; FDR, false discovery rate; DEG, differentially expressed genes; BTM, blood transcriptional modules.

MATERIALS AND METHODS

Vaccines

The vaccine strain *M. hyopneumoniae* F7.2C was grown in modified Friis medium (35) for 5 days at 37°C. The culture,

containing 5×10^8 color changing units (CCU)/ml, was inactivated by incubation with 4 mM binary ethyleneimine (BEI) under agitation at 37°C for 24 h. Subsequently, the BEI was neutralized by incubating the inactivated culture with 4 mM sodium thiosulfate under agitation at 37°C for 24 h. Inactivated bacteria were pelleted at 15,000 g for 40 min at 4°C and washed three times in 50 ml sterile phosphate buffered saline (PBS). The final pellet was resuspended in sterile PBS.

For this study, five adjuvant formulations were developed based on the association of particle-based delivery systems [liposomes, poly(lactic-co-glycolic acid) [PLGA] microparticles and a squalene-in-water emulsion (SWE)] with different immune stimulators. These included the Mincle agonist trehalose 6,6-dibehenate (TDB, Avanti, Alabaster, AL, USA), the STING ligand cyclic diadenylate monophosphate (c-di-AMP, produced at the Helmholtz Center for Infection Research, Braunschweig, Germany) and a combination of TLR ligands: TLR1/2 ligand Pam3Cys-SK4 (PAM, EMC Microcollections, Tübingen, Germany), TLR9 ligand CpG ODN SL03 (CpG, Eurofins Genomics, Les Ulis, France), and TLR7/8 ligand resiquimod (Chemdea, Ridgewood, NJ, USA).

Two cationic liposome formulations were produced, based on the thin lipid film method (36), and followed by extrusion: TDB was combined with dimethyl dioctadecylammonium (DDA) bromide to form Lipo_DDA:TDB, and c-di-AMP was encapsulated into 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and dimethylaminoethane-carbamoyl-cholesterol (DPPC:DC-Chol) cationic liposomes (37) to obtain Lipo_AMP. The TLR ligand selection was combined in different delivery systems: PLGA micro-particles, cationic liposomes and SWE. Cationic liposomes (DPPC:DC-Chol) and PLGA cationic micro-particles (combined to ethylaminoethyl-dextran) were produced with the thin lipid film and the double emulsion (W/O/W) methods (38), respectively. Pam3Cys-SK4 and resiquimod were encapsulated into both types of particles and CpG was later adsorbed to their surface to form the Lipo_TLR and PLGA_TLR formulations. Finally, for the SWE_TLR formulation, SWE [a squalene-based formulation developed and produced by the Vaccine Formulation Laboratory, and composed of 3.9% (w/v) squalene, 0.5% (w/v) Tween 80 and 0.5% (w/v) Span 85 (39)] was mixed with the same immune stimulators (PAM, CpG, and resiquimod).

For each formulation we measured the following physico-chemical characteristics: particle size (PS), polydispersity index (Pdi), and zeta potential (ZP), by means of dynamic light scattering for the liposomes and SWE, and laser diffraction for the micro-particles (Zetasizer Nano ZS and Mastersizer 3000, Malvern, UK). The amounts of immune-stimulators loaded into the Lipo_AMP and Lipo_TLR formulations were indirectly determined by the use of nanodrop (for c-di-AMP) and fluorescently labeled immune-stimulator (CpG-FITC and Pam-Rodhamine, Invivogen, San Diego, CA, USA) methods. The free immune-stimulators were separated from the liposomes by filtration using the Vivaspin® 500 centrifugal concentrator (PED membrane, MWCO 100 kDa, Sartorius, Göttingen, Germany) and then quantified as mentioned above (**Supplementary Table 1**) Antigen was mixed with the final product, and PS and ZP of the formulations were monitored over a period of 1 month.

The composition of each experimental vaccine is given in **Table 1**. The commercial vaccine employed was Hyogen® (CEVA Santé Animale, Libourne Cedex, France) representing a mineral oil emulsion with *Escherichia coli* J5 non-toxic LPS as immunostimulant and inactivated *M. hyopneumoniae* field isolate BA 2940-99 as antigen.

Animal Experiment

The study was performed after approval by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2016/91). Forty-two *M. hyopneumoniae*-free Rattlerlow-Seghers piglets (RA-SE Genetics NV, Ooigem, Belgium) were enrolled in the study. All animals were purchased from a herd that has been free of *M. hyopneumoniae* for many years based on repeated serological testing, nested PCR testing on tracheobronchial swabs, and absence of clinical signs and pneumonia lesions in the slaughter house. The piglets were weaned at 28 days of age and transported 4 days later to the experimental facilities of the Faculty of Veterinary Medicine, Ghent University, Belgium. They were housed in stables with absolute air filters for impending particles (HEPA U15) on both incoming and outgoing ventilation shafts and fed *ad libitum* with a non-antimicrobial-supplemented diet. On the day of arrival at the experimental facilities, the piglets were randomly allocated into six vaccination groups and one control group of six piglets each. Due to practical reasons, the piglets were vaccinated, sampled and euthanized over 2 consecutive days. After an acclimatization period of 6 days, the piglets of the vaccination groups were primo-vaccinated (D0; 39–40 days of age) intramuscularly (IM) into the right side of the neck with 2 mL vaccine. Additionally, group Lipo_DDA:TDB was vaccinated intradermally (ID) into the left side of the neck with 0.2 mL vaccine. The rationale for the ID injection of formulation Lipo_DDA:TDB was based on a previous report showing that CAF01, a liposome-based adjuvant containing similar immunomodulators, was able to induce mucosal immunity when administered this way (40). The piglets of the control group were injected IM into the right side of the neck with 2 mL sterile PBS. Two weeks later (D14), the piglets of the vaccination groups were booster vaccinated IM with 2 mL vaccine (all groups). The control group received 2 mL PBS IM. On D28 all piglets were euthanized.

Safety Parameters

The piglets were observed daily for at least 15 min from D-6 until D28 of the study. On the days of vaccine administration, the piglets were observed twice: shortly before (D0; D14) and 4 h after vaccination (D0+4h; D14+4h). For each piglet, clinical findings regarding body condition (skinny), behavior (depressed, unconscious), respiration (sneezing, coughing, abdominal breathing), digestion (diarrhea, vomiting), lameness and other remarkable findings were recorded. At necropsy (D28), lungs were macroscopically examined for the presence of lesions according to Hannan et al. (41). Subsequently, bronchoalveolar lavage (BAL) fluid was collected from one lung part by flushing the head bronchus with 20 mL sterile PBS, as previously described (15). From the BAL fluid, DNA was extracted using a commercial kit (DNeasy® Blood & Tissue

TABLE 1 | Composition of the experimental *M. hyopneumoniae* bacterins and their route of administration.

Vaccine formulation	Dose (mL)	Delivery system	Immune-stimulators (μg/dose)	Antigen dose (CCU)	Administration route	
					Primo	Booster
Lipo_AMP	2	DPPC:DC-Chol liposomes	C-di.AMP (100)	10^9	IM	IM
Lipo_TLR	2	DPPC:DC-Chol liposomes	Pam3Cys-SK4/CpG ODN SL03/resiquimod (80/80/80)	10^9	IM	IM
PLGA_TLR	2	PLGA micro-particles (combined to ethylaminoethyl-dextran)	Pam3Cys-SK4/CpG ODN SL03/resiquimod (80/80/80)	10^9	IM	IM
SWE_TLR	2	squalene-in-water emulsion	Pam3Cys-SK4/CpG ODN SL03/resiquimod (80/80/80)	10^9	IM	IM
Lipo_DDA:TDB	IM 2 ID 0.2	DDA liposomes	TDB (500)	IM 10^9 ID $2x10^8$	IM+ID	IM

CCU, color changing units; IM, intramuscular; ID, intradermal; DPPC:DC-Chol, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and dimethylaminoethane-carbamoyl-cholesterol; c-di-AMP, bis-(3′,5′)-cyclic dimeric adenosine monophosphate; PLGA, poly(lactic-co-glycolic acid); DDA, dimethyl dioctadecylammonium; TDB, trehalose 6,6′-dibehenate.

kit, Qiagen, Venlo, The Netherlands) and a nested PCR for the detection of *M. hyopneumoniae* DNA was performed according to Stärk et al. (42).

The pigs were weighed on the day of primo-vaccination (D0) and at euthanasia (D28). Average daily gain (ADG) in g/pig/day was calculated according to Sacristán et al. (43).

Rectal body temperature was measured shortly before and 4 h after vaccine administration, then daily until 4 days post-vaccine administration, and on D7, 10, 21, 24, and 28 of the study. This was based on the guidelines on safety evaluation of veterinary vaccines written in the European Pharmacopeia 8.0.

Injection site reactions (ISR) were evaluated shortly before vaccination, 4 h after vaccination and then daily from D1 to D28 using the scoring system explained in **Supplementary Table 2**. Scores could range from 0 to 3 with 0 = normal, 1 = mild, 2 = moderate, and 3 = severe. At euthanasia (D28), tissue samples from the injection site were collected from all study animals for histopathological examination. All IM and ID injection sites were marked with a permanent pen upon vaccination. Out of the marked area a tissue sample of approximately 2 cm^2 with a depth of 5 cm (IM injection site) or 3 cm (ID injection site) was removed in an angle of 90° to the skin. A tissue sample with a dimension of 2 × 2 × 3 cm from the left side of the neck was collected as described above from the pigs of the control group to serve as a control for the ID injection sites. The tissues were fixed immediately after sampling in 10% neutral formalin. After fixation, tissue blocks were sectioned from the samples, embedded in paraffin and histological slides were stained with hematoxylin and eosin. Each injection site sample was evaluated using light microscopy and an overall score ranging from 0 to 3 (0 = not detected, 1 = mild, 2 = moderate, and 3 = severe) was given. This score took into account the presence and degree of hemorrhage, blood resorption, necrosis, inflammation (acute and chronic), angiogenesis, and proliferation of connective tissue.

Serology

Before primo-vaccination (D0), on D7, on the day of booster vaccination (D14) and at euthanasia (D28), serum samples were collected and analyzed for the presence of antibodies against *M. hyopneumoniae* with a commercial blocking ELISA (IDEIA™ *Mycoplasma hyopneumoniae* EIA kit, Oxoid Limited, Hampshire, UK) according to the manufacturer's instructions. Samples with optical density (OD) lower than 50% of the average OD of the buffer control were considered positive. Samples with OD-values equal or bigger than 50% of the average OD of the buffer control were classified as negative.

Immunoglobulin (Ig) G and IgA isotypes of the *M. hyopneumoniae*-specific antibodies in serum were determined with an in-house indirect ELISA. Briefly, Nunc Maxisorb® flat-bottom 96 well plates (eBioscience, San Diego, CA, USA) were coated overnight at room temperature with Tween 20-extracted *M. hyopneumoniae* antigens (44). After blocking with PBS containing 0.05% Tween 20 and 1% BSA for 2 h at 37°C, plates were washed three times with PBS + 0.05% Tween 20 and serum diluted 1:200 and 1:100 was added for the detection of IgG and IgA, respectively. After incubating for 30 min at 37°C, plates were washed again, and peroxidase-labeled goat anti-porcine polyclonal IgG diluted 1:60,000 and IgA diluted 1:20,000 (Bethyl Laboratories, Montgomery, TX, USA) were added. Plates were incubated again for 30 min at 37°C, washed and 3,3′5,5′-tetramethylbenzidin substrate (Sigma-Aldrich, Saint Louis, MO, USA) was added. After incubating for 10 min, the reaction was stopped with 2 N HCl and the OD was measured at 450 nm. All samples were tested in duplicate. To relatively quantify the antibody levels a standard curve was made using two-fold serial dilutions of a positive reference serum corresponding to defined arbitrary units (1:800 dilution defined as 1 unit). The interpolation from the standard curve employed non-linear regression with least square fits using Graphpad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA).

M. hyopneumoniae-Specific Antibodies in Bronchoalveolar Lavage (BAL) Fluid

The BAL fluid collected on D28 was analyzed undiluted for the presence of *M. hyopneumoniae*-specific IgA antibodies using peroxidase-labeled goat anti-porcine polyclonal IgA (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:80,000 in an in-house indirect ELISA as described above. A cut-off was calculated as mean OD-value from the control animals plus three times

the SD and established at an OD-value of 0.098. Samples with OD-values higher than the cut-off were considered positive and samples equal to or below the cut-off were considered negative.

T Cell Assays

Shortly before the booster vaccination on D14 and on the day of euthanasia (D28), blood samples were taken from each animal to assess the primary and secondary T cell-specific responses against *M. hyopneumoniae*. For each animal, samples were restimulated in triplicate cultures and analyzed separately. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using a ficoll-plaque density gradient (1.077 g/L, GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA) and plated in 12-well plates at 5×10^6 cells/well in 1 ml of AIM-V medium (Gibco™, ThermoFisher Scientific, Waltham, MA, USA). Subsequently, the cells were restimulated *in vitro* overnight (18 h) with 6.25×10^7 CCU/mL of *M. hyopneumoniae* F7.2C bacterin. For the last 4 h of stimulation, we added Brefeldin A (eBioscience, San Diego, CA, USA) in each well to inhibit cytokine release and allow intra-cellular detection of cytokines by flow cytometry (FCM). Concanavalin A stimulation (10 μg/mL, Sigma-Aldrich, Saint Louis, MO, USA) was employed as a positive control. Cells were then harvested and the cytokine production of T cell populations was determined by FCM, using a 5-step 6-color staining protocol. Cells were first incubated with the LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The cells were then incubated with anti-CD4 (clone 74-12-4, Southern Biotech, Birmingham, AL, USA) and anti-CD8β (clone PG164A, WSU, Pullman, WA, USA) antibodies, and subsequently with the corresponding secondary antibodies: anti-mouse IgG2b AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) and anti-mouse IgG2a PE-Cy7 (Abcam, Cambridge, UK), respectively. Following surface staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were finally incubated with directly coupled anti-human TNF-α AlexaFluor 647 (clone MAb11, BioLegend, San Diego, CA, USA), anti-pig IFN-γ PerCP-Cy5.5 (clone P2G10, Becton Dickinson, Franklin Lakes, NJ, USA), and anti-human IL-17A PE (clone SCPL1362, Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry acquisition was performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) and the results were further analyzed with the FlowJo™ software (Tree Star Inc., Ashland, OR, USA).

Vaccine-Induced Transcriptional Responses

Blood samples were collected on D0, D1, and D7 for RNA preparation (2.5 ml in PAXgene® Blood RNA Tubes, Becton Dickinson, Franklin Lakes, NJ, USA). RNA was extracted using the Paxgene® Blood RNA kit (Qiagen, Venlo, The Netherlands) and the RNA quality was controlled with a Fragment Analyzer. All samples were found to have good quality [RNA integrity number (RIN) > 8] and were sequenced using an Illumina® HiSeq 3000 sequencer (Illumina, San Diego, CA, USA). The quality of the reads was assessed using FastQC v. 0.11.2[1] The reads were mapped to the *Sus scrofa* reference genome (Sscrofa_11.1) with HISAT2 v. 2.1.0 (45). Feature Counts from Subread v. 1.5.3 was employed to count the number of reads overlapping with each gene, as specified in the Ensembl annotation build 91. The RNAseq data are available in the European Nucleotide Archive[2] under the accession number PRJEB30361.

The Bioconductor package DESeq2 v. 1.18.1 was used to test for differential gene expression between the different time points for each vaccine separately (46). Our specific interest was to identify genes where the change between two time points was different in vaccinated animals compared to the controls. Therefore, a two-factorial model was used, including the factors time point and group (vaccine vs. control), and their interaction. The genes were then ranked based on the P-values for the interaction term for a "ranked gene set enrichment analysis" (GSEA) (47) using the BTM as defined by Li et al. (48).

The BTM were adapted to the pig by replacing human genes with their pig homologs. This step involved extensive manual curation. The final lists of genes for each module can be found in the **Data Sheet 1**.

To compare the module activity of the different vaccines, all modules with a false discovery rate (FDR) $q < 0.1$ were used. In GSEA, a cut-off of 0.25 is recommended but in this study a cut-off of 0.1 was selected to reduce the amount of BTM changing over time. Heat maps were created reflecting the modular activity calculated as the negative natural logarithm of the *P*-value. For negative enriched BTM, this was multiplied with −1 to obtain a positive value. The rationale of this was to obtain a value reflecting both the enrichment of a module and its statistical significance.

Correlation Analyses of BTM and Vaccine-Induced Adaptive Immune Responses

To get more insight in the immunomodulation toward a potent immune response, BTM were correlated with the vaccine-induced adaptive immune responses (antibodies, *M. hyopneumoniae*-specific $INF\gamma^+TNF^+$ CD4 T cells and CD8 T cells). To this end, single-sample (ss) GSEA scores were first calculated to transform a single sample's gene expression profile to a gene set (BTM) enrichment profile[3] as described in Barbie et al. (49). Subsequently, the time-dependent changes in ssGSEA values for each BTM were determined as the ratio of D1:D0, D7:D0, and D1:D7 ssGSEA values. These ratios were then correlated to the immune response values using Pearson's correlation coefficient. In order to obtain sufficient values, the data from all vaccinated animals (controls excluded) was used. Only correlation coefficients with $P < 0.05$ were considered.

[1] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[2] www.ebi.ac.uk/ena
[3] http://software.broadinstitute.org/cancer/software/genepattern/modules/docs/ssGSEAProjection/4

Statistical Analyses

Fisher's exact tests were performed to analyse differences in the number of animals with ISR and histopathological findings (irrespective of type) at the injection site between the control group and the vaccinated groups. A Bonferroni correction for multiple tests was applied. Rectal temperature values were averaged for the following periods: D1-3, D4-14, D15-17, and D18-28 to distinguish between systemic reactions shortly after vaccination (D1-3; D15-17) and systemic reactions developed later on (D4-14; D18-28). Rectal temperature and ADG were not normally distributed according to the Shapiro-Wilk's test, and Mann-Whitney U tests were run to analyse differences between the control and vaccinated groups in ADG, rectal temperature measured 4 h after vaccination (D0+4h; D14+4h) and during the following periods: D1-3, D4-14, D15-17, D18-28. The Bonferroni method was applied to correct for multiple comparisons. For the quantitative antibody ELISA and T cell data, a two-way ANOVA was employed using the factors vaccine and time. Tukey's or Dunnett's tests were used to correct for multiple comparisons, respectively. Statistical analyses of clinical variables were conducted in SPSS 24 for Windows (IBM, Armonk, NY, USA) and for immune response data using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Significance is indicated as $^{*}P \leq 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Safety of the Vaccines

To evaluate the safety of the vaccines the general health, ADG and rectal temperature of the piglets was closely monitored. Diarrhea, which sometimes resulted in skinny pigs, was the most frequent clinical finding observed in all groups (Lipo_DDA:TDB: 2/6; Hyogen and PLGA_TLR: 4/6; control, Lipo_TLR, SWE_TLR: 5/6; Lipo_AMP: 6/6; **Supplementary Figure 1B**). As it was mostly seen during the acclimatization period and started the day after arrival, it was diagnosed as post-weaning diarrhea. All pigs were treated once with 5 mg enrofloxacin per kg body weight (Floxadil® 50 mg/mL, Emdoka, Hoogstraten, Belgium) IM in the hind leg and responded well on treatment. Arthritis (swollen joints) was also observed (control, Lipo_AMP, Lipo_TLR, PLGA_TLR: 1/6; Hyogen: 3/6) and cases occurred during the whole study period (**Supplementary Figure 1C**). Bursitis was recorded for one pig in groups Lipo_TLR, SWE_TLR and Hyogen, and lameness for one pig in groups Lipo_AMP and Lipo_DDA:TDB (**Supplementary Figures 1D–E**). Behavior and respiration were normal throughout the entire study, except for one pig of the PLGA_TLR group that showed severe abdominal breathing following blood sampling on D14. At necropsy (D28), none of the pigs had macroscopic lung lesions and no *M. hyopneumoniae* DNA was detected in BAL fluid. The vaccinated groups did not differ in ADG compared to the control group (data not shown). Four hours after primo- and booster vaccination (D0+4h, D14+4h), rectal temperatures of groups SWE_TLR and Hyogen were significantly higher compared to the control group ($P \leq 0.05$). Rectal temperatures from Lipo_AMP and Lipo_TLR were also increased over the physiological threshold (>40°C) 4 h after primo- and booster vaccination. However, this increase was only statistically significant compared to the control group at D0+4h and D14+4 for groups Lipo_TLR and Lipo_AMP, respectively ($P \leq 0.05$). A slight increase, although not statistically significant, was observed for PLGA_TLR 4 h after primo-vaccination. Group means were back to normal 1day after vaccination and all remained within normal physiological levels during the remainder of the trial (**Supplementary Figure 1F**).

The presence and severity of ISR was recorded daily and a histopathological examination of each injection site was performed at the end of the study (D28). No ISR were seen in the control group and group PLGA_TLR. Overall mild and transient ISR were observed in one pig of group SWE_TLR, and in two pigs of each of the groups Lipo_AMP, Lipo_TLR, and Hyogen. In the group Lipo_DDA:TDB three pigs showed a moderate but transient ISR at the IM injection site. However, at the ID injection site, all pigs showed a prolonged mild to moderate ISR which lasted until the end of the study in 4/6 pigs. A more detailed overview of the duration of the ISR and their severity is given in **Supplementary Table 3**. Histopathological examination of the injection site at D28 revealed an overall severe foreign body reaction with chronic inflammation, angiogenesis, and proliferation of connective tissue in 5/6 ID injection site samples from group Lipo_DDA:TDB. Mild (moderate for one pig in group SWE_TLR) focal chronic inflammation was observed in all IM injected groups. Mild to moderate hemorrhage was also observed in all IM injected groups. This was probably caused by the sampling itself as it was most of the time located at the borders of the collected tissue. The results of the histopathological examination of the injection sites are represented in **Supplementary Table 4**.

M. hyopneumoniae-Specific Antibody Responses

According to the commercial blocking ELISA (Oxoid; **Supplementary Table 5**), all pigs from the control group remained serologically negative for *M. hyopneumoniae* throughout the study. On day 28 of the study, all the animals from groups Lipo_AMP, Lipo_TLR, SWE_TLR, Lipo_DDA:TDB, and Hyogen were seropositive. From the PLGA_TLR group, only two out of six pigs seroconverted at D28.

To quantify serum IgG levels in arbitrary units we used an in-house indirect ELISA with a positive reference serum as a standard (**Figure 1**). At D28, groups Lipo_AMP, SWE_TLR, Lipo_DDA:TDB, and Hyogen were statistically different from the control group. The Lipo_DDA:TDB formulation induced the highest IgG response, followed by the Hyogen and Lipo_AMP formulations. Group Lipo_TLR was not significantly higher than the control group, although we could detect *M. hyopneumoniae*-specific IgG antibodies in all animals from this group. In the PLGA_TLR group, only one animal appeared to react. No *M. hyopneumoniae*-specific IgA antibodies were observed for any of the groups at any time point in the serum. Only one animal from the SWE_TLR group was positive for *M. hyopneumoniae*-specific IgA in BAL fluid on D28 (**Supplementary Table 3**).

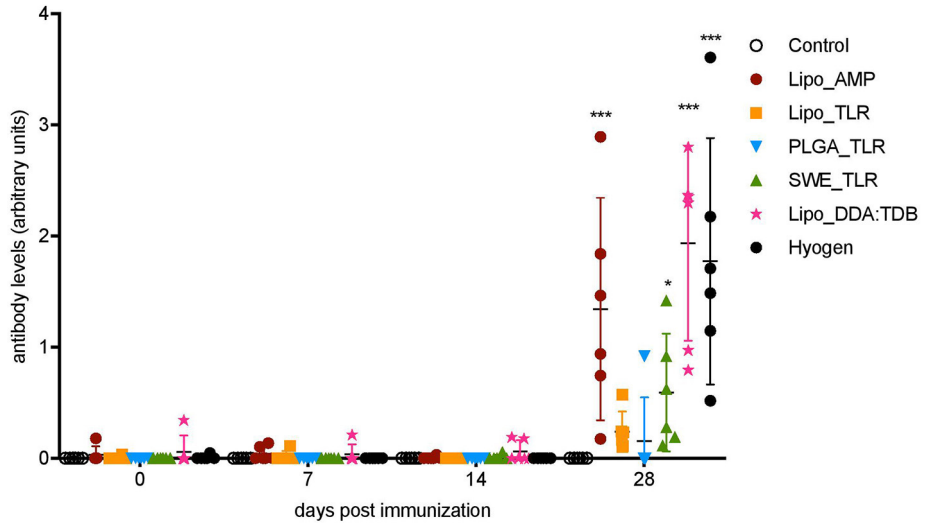

FIGURE 1 | Serum antibody levels following vaccination of pigs with vaccine candidates. *M. hyopneumoniae*-specific IgG antibodies induced by the five novel vaccines and one commercial vaccine listed in the legend were determined by indirect ELISA. Six animals per group received two injections in 14 days interval. Individual animals are shown. Significance was calculated using two-way ANOVA followed by Tukey's test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

T Cell Responses

The results of the *M. hyopneumoniae*-specific T cell responses after primo-vaccination (D14) are presented in **Figures 2A–C**. No significant group differences were found for the percentage of cytokine-producing T cells in the peripheral blood compartment. Nevertheless, as antigen-specific T cells are transient in the blood, a negative result cannot be interpreted as a lack of T cell response. In fact, a few animals appeared to respond (defined as being above the 99% confidence interval (CI) of the control group) indicating some degree of T cell priming in certain groups. This was found in particular in the groups SWE_TLR, Lipo_DDA:TDB and Hyogen with three animals above this threshold for the $TNF^+IFN\text{-}\gamma^+$ double positive CD4 (Th1) cells (**Figure 2A**). For the $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells, two animals were above the threshold in the PLGA_TLR, SWE_TLR, and Hyogen groups (**Figure 2C**).

At D28, the SWE_TLR and Lipo_DDA:TDB groups were significantly higher than the control group for the percentage of $CD4^+$ $TNF^+IFN\text{-}\gamma^+$ T cells (**Figure 2D**) and the PLGA_TLR group was significantly higher than the control group for the percentage of $CD4^+IL17A^+$ (Th17) cells (**Figure 2E**). For the percentage of $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ T cells, groups Lipo_AMP and lipo_DDA:TDB were significantly higher compared to the control animals (**Figure 2D**). Despite the lack of statistical significance, other vaccines also appeared to have induced specific T cell immunity in some animals. For the $CD4^+$ $TNF^+IFN\text{-}\gamma^+$ cells, three animals were above the 99% CI threshold in the Lipo_AMP group and two in the Hyogen group. For the $CD8^+$ $TNF^+IFN\text{-}\gamma^+$ cells, two pigs were above the 99% CI threshold in the Lipo_TLR group, five in the SWE_TLR group and three in the Hyogen group (**Figures 2D–F**).

When focusing on $TNF^-IFN\text{-}\gamma^+$-producing T cells, we found a high level of non-specific responses at both D14 and D28 in the unvaccinated group which "masked" the vaccine induced responses (**Supplementary Figure 2**). Only Lipo_DDA:TDB induced a significant level of $CD4^+$ $TNF^+IFN\text{-}\gamma^-$-producing T cells at D28 (**Supplementary Figure 3**).

In conclusion, the vaccines SWE_TLR and Lipo_DDA:TDB induced a statistically significant Th1 driven T cell response. In the groups receiving the Hyogen and Lipo_AMP formulations, despite a trend suggesting stimulation of Th1 responses, the differences were not statistically significant in the current setting. Interestingly, the PLGA_TLR formulation was the only vaccine candidate which significantly induced a Th17 response, although only 3/6 animals in this group were above the threshold.

Blood Transcriptional Modules Correlating to Vaccine-Induced Adaptive Immune Responses

In order to shed light on the immunological perturbations associated with adaptive immune responses, changes in transcriptional modules expression were correlated to the immune responses shown in **Figures 1**, **2**.

For the early transcriptional responses (determined as modular changes between D0 and D1), a total of seven inflammatory, eight myeloid cell, three DC/antigen presentation and one IFN type I BTM correlated positively with the antibody response. Interestingly, none of these modules correlated with the CD4 T cell response, but some with the CD8 T cell response. For the late transcriptional responses (determined as modular changes between D1 and D7), a negative correlation was found for many BTM belonging to the families of modules reflecting innate immune responses. This was found again mainly for the antibody and CD8 T cell responses (**Figure 3**).

Main positive correlations of the CD4 T cell response were the D0 to D7 changes in cell cycle BTM (**Figure 4**). For the change of

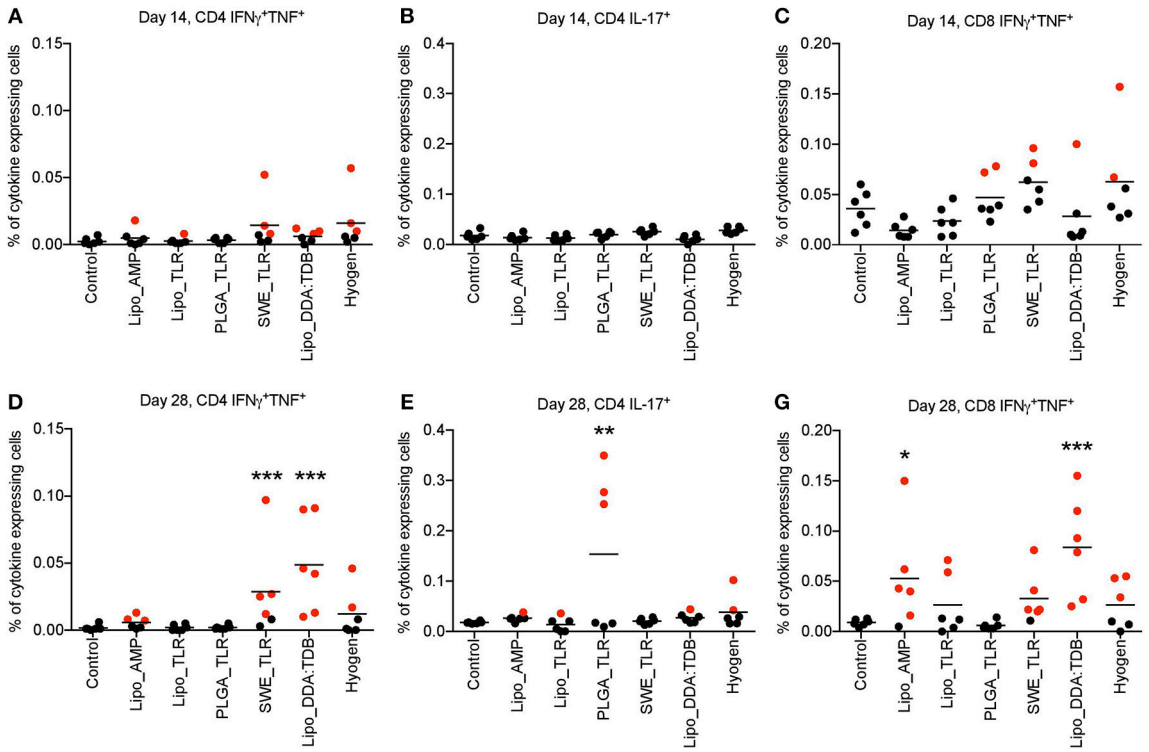

FIGURE 2 | *M. hyopneumoniae*-specific T cell responses induced following vaccination of pigs with vaccine candidates. Six animals per group were prime-boost vaccinated on D0 and D14. At D14 and D28, *M. hyopneumoniae*-specific T cells induced by the tested vaccines listed in the legend were determined by *in vitro* restimulation of PBMC from vaccinated animals followed by intracellular cytokine staining and multicolor flow cytometry. Following doublet exclusion, live cells were gated and the percentage of $IFN\gamma^+TNF^+$ double positive $CD4^+$ **(A,D)** and $CD8\beta^+$ **(C,F)** T cells as well as $IL\text{-}17A^+$ CD4 T cells **(B,E)** was determined. The mean values obtained from triplicate cultures for individual animals are shown. Positive animals are marked in red (defined as being above the 99% CI of the control group). Significance was calculated using two-way ANOVA followed by Dunnett's test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). PBMC, peripheral blood mononuclear cells.

cell cycle BTM between D1 and D7, we also found many modules correlating with antibody and T cell responses.

T/NK cell BTM upregulation between D0 and D7 correlated well with CD8 T cell responses. The induction of these BTM also correlated with antibody levels between D1 and D7 (**Figure 4**).

Transcriptional Profiling of Vaccines

To better understand differences in the induction of immune responses between the vaccines, we next performed a transcription profiling. From the reads obtained, we first calculated the differentially expressed genes (DEG) using DSeq2, and then employed a two-factorial model, including the factors time point and group (vaccine vs. control), to identify genes differing between two time points in vaccinated animals compared to the controls. Next, we used ranked GSEA analyses using BTM as gene sets and ranked DEG between D0 and D1, D0 and D7, and D1 and D7 of each vaccine group. All data are shown in **Figures 5–8** and **Supplementary Figures 4, 5**, and summarized in **Table 2**.

Inflammatory Responses

From D0 to D1, the Lipo_AMP formulation induced the highest number of inflammatory BTM, followed by the groups SWE_TLR and Lipo_DDA:TDB (**Figure 5**). Interestingly, in the PLGA_TLR group, no inflammatory BTM were induced and some even showed a downregulation. For the D0 to D7 comparison, again groups Lipo_AMP and SWE_TLR showed the highest upregulation of these BTM. For the D1 to D7 comparison, we found a downregulation of inflammatory modules in the Lipo_AMP, Lipo_TLR, and the Hyogen groups but not in the Lipo_DDA:TDB group, which still had BTM related to platelet activation overexpressed. In the PLGA_TLR group, eight BTM were upregulated indicating a delayed innate immune response. In summary, the three vaccines which induced significant T cell responses in terms of IFNγ/TNF secreting cells as well as antibody responses were those with the strongest positive early upregulation of inflammatory BTM, confirming the results obtained using the correlation analysis (**Figure 3**).

IFN Type I Responses

With respect to IFN type I BTM, only vaccines which contained IFN inducers such as c-di-AMP (Lipo_AMP) and CpG (Lipo_TLR, SWE_TLR) induced an early IFN type I BTM response. The PLGA_TLR formulation contained the same TLR cocktail as SWE_TLR and Lipo_TLR, but was unable to induce such responses (**Figure 5**).

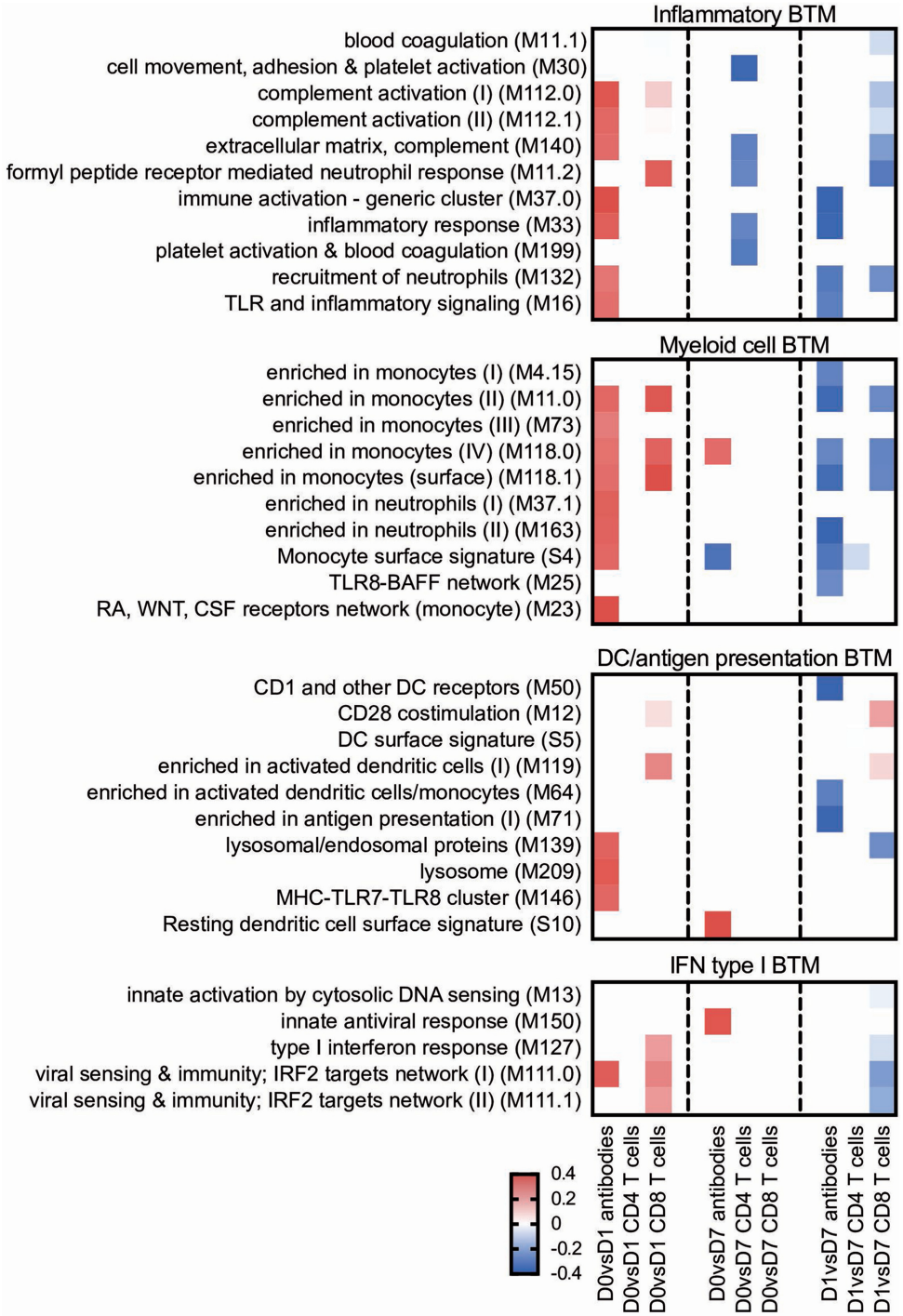

FIGURE 3 | Innate immune response BTM correlating with adaptive immune responses. Time-dependent changes in scores for BTM were determined using ssGSEA and then correlated to antibodies, *M. hyopneumoniae*-specific $INF\gamma^{+}TNF^{+}$ CD4 T cells and CD8 T cells. Pearson correlation coefficients for the BTM changes from D0 to D1 (D0 vs. D1), from D0 to D7 (D0 vs. D7), and from D1 to D7 (D1 vs. D7) are shown as heat maps. A $P < 0.05$ was used as cut-off. Red colors indicate positive and blue negative correlations. The BTM were grouped into inflammatory, myeloid cell, DC/antigen presentation and IFN type I BTM as previously described (34). BTM, blood transcriptional modules.

Myeloid and DC/Antigen Presentation Responses

All vaccines with the exception of PLGA_TLR induced an early (D0 to D1) myeloid cell response (**Figure 6**). The number of BTM being modulated was the highest in the Lipo_AMP group, followed by the groups SWE_TLR, Lipo_DDA:TDB, and Hyogen (the latter two being very similar). The PLGA_TLR

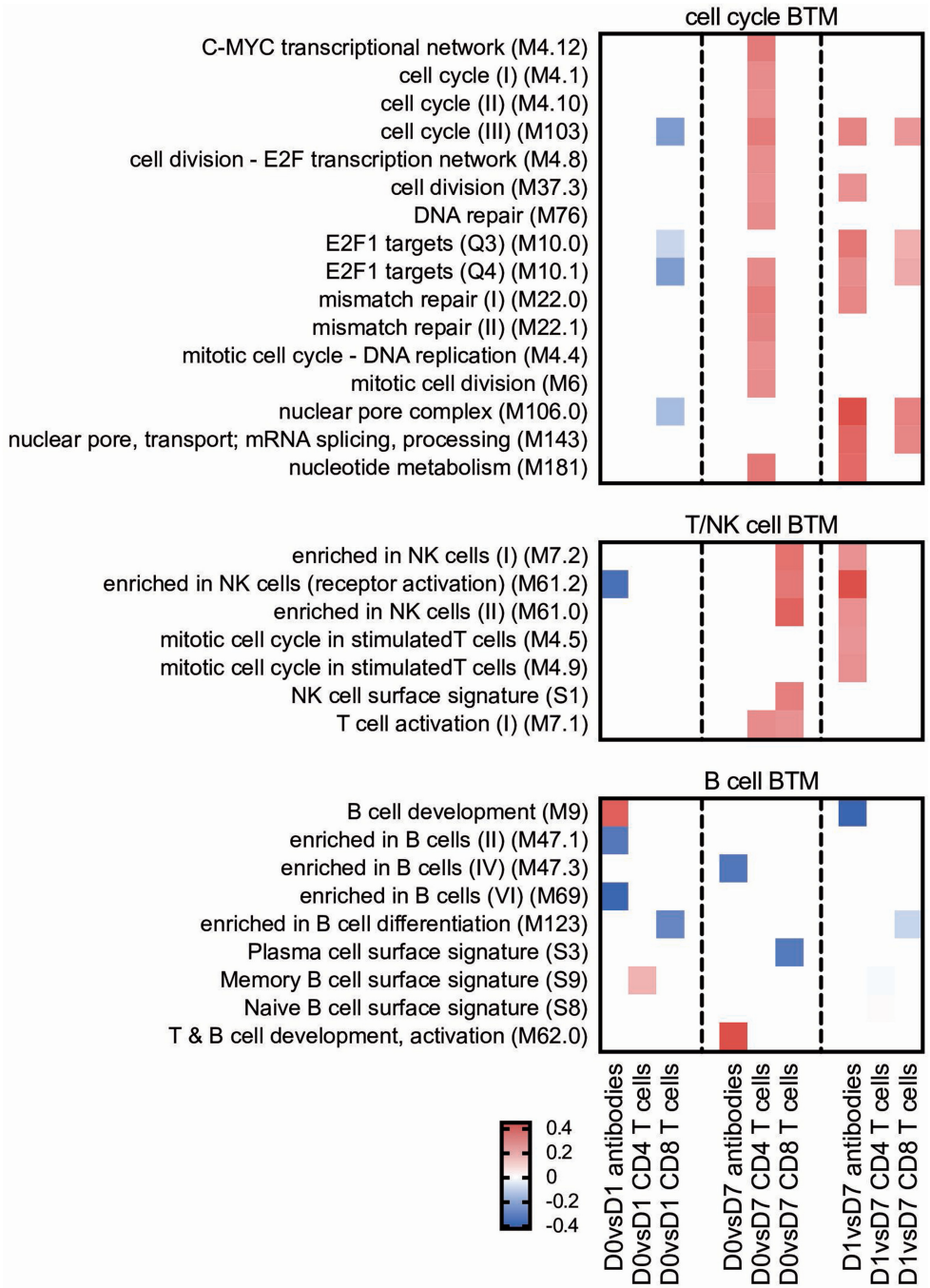

FIGURE 4 | Cell cycle and lymphocyte BTM correlating with adaptive immune responses. Time-dependent changes in scores for BTM were determined using ssGSEA and then correlated to antibodies, *M. hyopneumoniae*-specific $INF\gamma^{+}TNF^{+}$ CD4 T cells and CD8 T cells. Pearson correlation coefficients for the BTM changes from D0 to D1 (D0 vs. D1), from D0 to D7 (D0 vs. D7) and from D1 to D7 (D1 vs. D7) are shown as heat maps. A $P < 0.05$ was used as cut-off. Red colors indicate positive and blue negative correlations. The BTM were grouped into cell cycle, T/NK cell and B cell BTM as previously described (34). BTM, blood transcriptional modules.

formulation actually had a negative influence on myeloid cell BTM response. Only Lipo_AMP, Lipo_TLR, and Hyogen induced a clear downregulation of these BTM from D1 to D7. This was interesting considering that a late D1 to D7 downregulation of myeloid cell BTM was found to strongly correlate with antibody and CD8 T cell responses (**Figure 3**). In summary, the vaccines which induced good adaptive immune responses were also those which induced an early induction of many myeloid cell BTM.

The Lipo_AMP formulation was found to be the most potent to induce BTM relating to DC and antigen presentation from D0 to D1. Similar to the myeloid cell BTM, DC/antigen

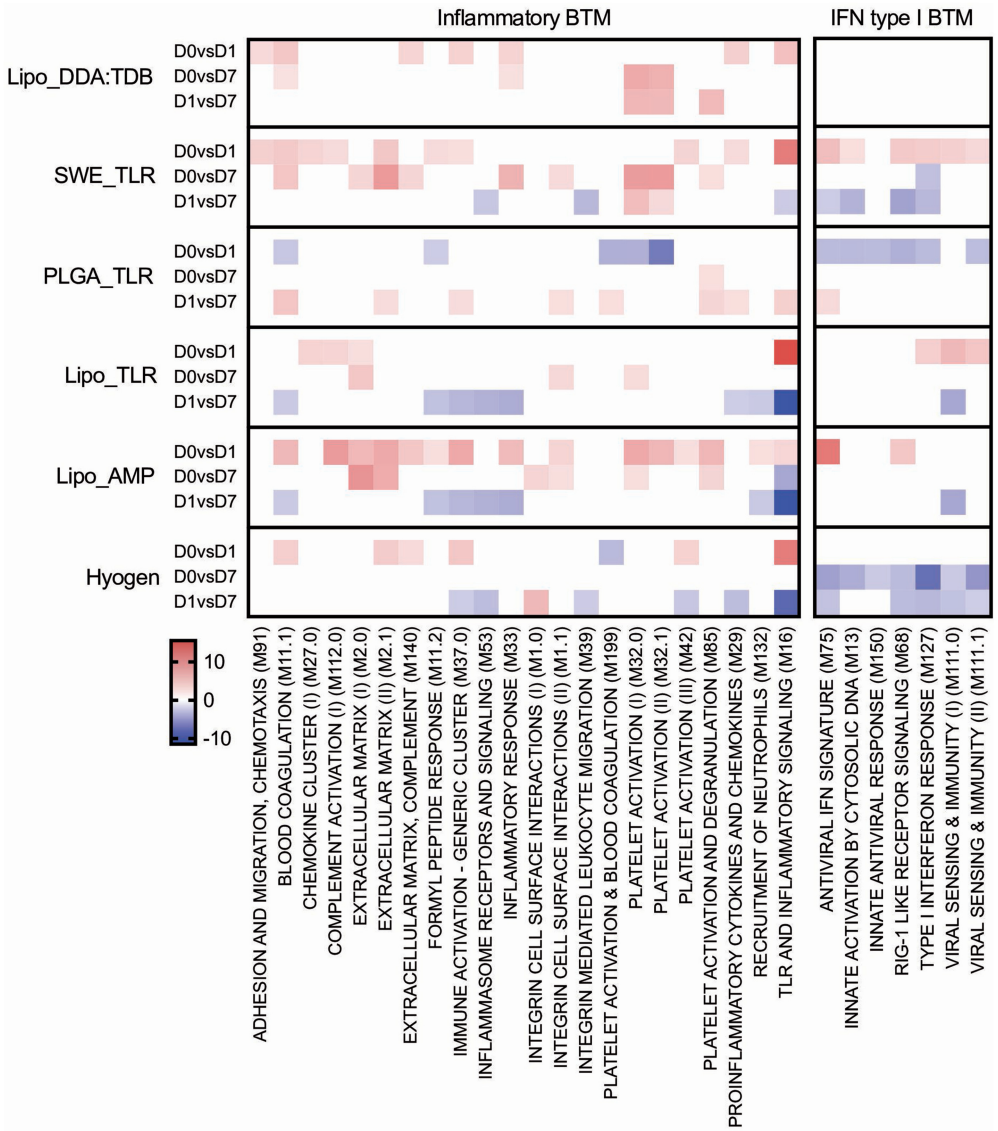

FIGURE 5 | Inflammatory and IFN type I BTM induced by vaccines. The heat maps show the vaccine-dependent induction of BTM activity determined for D0 to D1 (D0 vs. D1), for D0 to D7 (D0 vs. D7), and for D1 to D7 (D1 vs. D7) changes in the modules. The values shown were calculated by −log(P-value)*1 for positively enriched BTM and as −log(P-value)* −1 for negatively enriched BTM. A cut-off of an FDR of $q < 0.1$ was employed. Red colors indicate BTM upregulation and blue downregulation. BTM, blood transcriptional modules.

presentation BTM were downregulated from D1 to D7 by the formulations Lipo_AMP and Lipo_TLR, and to a lower extent by the Hyogen vaccine.

Cell Cycle/Proliferation

The Lipo_DDA:TDB and SWE_TLR vaccines were found to downregulate, while PLGA_TLR upregulated cell cycle BTM from D0 to D1 (**Figure 7**). The two liposomal formulations Lipo_AMP and Lipo_TLR had a clear positive effect on these BTM at later time points (D0 to D7 and D1 to D7). Interestingly, the correlation analyses demonstrated a clear association between the late (D0 or D1 to D7) upregulation of these BTM and adaptive immune responses (**Figure 4**).

B Cell BTM and T/NK Cell BTM

The Lipo_AMP, SWE_TLR, and Hyogen vaccines had an overall negative effect on the early expression (D0 to D1) of B-cell BTM (**Figure 8**). The SWE_TLR and Hyogen formulations were those to strongly induce these BTM at later time points (D1 to D7). Common BTM between the strong vaccines in terms of antibody responses were plasma cells and immunoglobulin (M156.0 and M156.1), which were overexpressed from D1 to D7. However, these BTM were not found significant in the correlation analyses (**Figure 4**).

For the T cell/NK cell BTM, a variable early downregulation by the more immunogenic vaccines Lipo_AMP, SWE_TLR, Lipo_DDA:TDB, and Hyogen was found. Only the liposomal

TABLE 2 | Overview of the immune responses induced by the *M. hyopneumoniae* bacterins.

Vaccine formulation	Ab response (D28)	Th1 response (D14/D28)	Th17 response (D14/D28)	Early inflam. BTM	Early IFN type I BTM	Early myeloid cell/DC BTM	Late cell cycle BTM	Late T/NK-cell BTM	Late Ig BTM
Lipo_AMP	++	+	–	+++	+	+++	++	++	++
Lipo_TLR	+	+	–	+	+	+	++	++	++
PLGA_TLR	–	–	+	–	–	–	–	–	–
SWE_TLR	+	++	–	++	++	++	–	–	+++
Lipo_DDA:TDB	++	+++	–	+	–	++	–	–	++
Hyogen	++	+	–	+	–	++	–	–	++

Six animals per group were prime-boost vaccinated on D0 and D14. Ab, antibody; Th, T helper; BTM, blood transcriptional modules; early, upregulation from D0 to D1; late, upregulation from D1 to D7; –, none to weak; +, moderate; ++, strong; +++, very strong.

formulations Lipo_AMP and Lipo_TLR induced a late D0/D1 to D7 upregulation of these modules, although many of those modules correlated to antibody and T cell responses (**Figure 4**).

DISCUSSION

The present study assessed the safety and performed a detailed immunological profiling of five novel *M. hyopneumoniae* bacterin formulations. We included as well the commercial vaccine Hyogen® in our study. Hyogen® is a recently developed bacterin based on a virulent *M. hyopneumoniae* field isolate with a TLR4 ligand as immunostimulant, and in that way comparable with our experimental bacterin formulations.

In terms of side effects, formulations Lipo_AMP, Lipo_TLR, and SWE_TLR induced a significant but transient increase in rectal body temperature shortly (4 h) after vaccination. This was also observed for the Hyogen® vaccine, and comparable observations were made by Llopart et al. (50) after two shot vaccination against *M. hyopneumoniae* with the commercial vaccine Mypravac suis® (HIPRA, Amer, Spain). Fever beginning a few hours after vaccination and persisting for 24 to 36 h is the result of an excessive induction of pro-inflammatory cytokines by the vaccine (51, 52). Systemic reactions of such kind are commonly reported and considered as "normal toxicity" associated with vaccination (52).

Overall, the ISR occasionally observed in all IM vaccinated groups and at the IM injection site of group Lipo_DDA:TDB were mild and resolved quickly. Transient redness and swelling at vaccination sites were also reported in other *M. hyopneumoniae* vaccination studies (53–55). Such local reactions often occur after parenteral administration of adjuvanted vaccines and are tolerated in terms of safety (52). Microscopically, mild focal chronic inflammation was observed in all IM injected groups, including the control group, indicating that these findings were probably caused by the tissue damage due to needle insertion and injection of fluid, and not by the administered vaccine formulation. Nevertheless, prolonged mild to moderate ISR were observed in all pigs from group Lipo_DDA:TDB at the ID injection site and histopathological examination of this injection site at D28 of the study showed a severe foreign body granuloma in five pigs. Local reactions of such kind could result in carcass trim losses at slaughter and are therefore considered to be a relevant adverse side effect of vaccination (51, 56). The transient ISR at the IM injection site of this vaccine group suggests that the prolonged and rather severe ISR is at least partially due to the ID administration. However, this cannot be stated with certainty as there was no control group ID injected with sterile PBS.

Two weeks after booster vaccination, the commercial vaccine Hyogen® as well as the vaccines Lipo_DDA:TDB and Lipo_AMP induced a strong humoral response. Vaccine formulations SWE_TLR and Lipo_TLR generated a moderate serological response, whereas for the PLGA_TLR formulation only two animals seroconverted. Nevertheless, as we do not know the antigen payload of the Hyogen® vaccine, we cannot directly compare its efficacy to the experimental vaccines. Although systemic antibodies are considered to play a minor role in protection against EP (5, 13), high levels of serum antibodies induced by vaccination can be an easy and practical tool to confirm successful vaccination in the field (57). It can also be expected that high levels of IgG will only be induced with significant induction of Th cell activation. We only found lgA antibodies in BAL fluid of one pig injected with the SWE_TLR vaccine. This is not surprising considering the parenteral vaccine administration, and is in line with previous studies showing IgA in BAL fluid of vaccinated pigs only after challenge (13, 14). Future studies are required to investigate the potential of adjuvants to induce both local and systemic immune responses after mucosal application of the vaccine. For example, this has been achieved for inactivated viruses using nanoparticle-based delivery (58, 59). Nevertheless, the absence of detectable IgA antibodies in BAL fluid from vaccinated pigs does not exclude priming of the immune system for such responses as vaccinated animals had higher mucosal IgA responses compared to unvaccinated animals following challenge (13, 14). Although we did not measure *M. hyopneumoniae*-specific IgG in BAL fluid, it can reach the alveolar lumen by transudation from the blood and might also play a role in protection against disease. In fact, the implementation of the human parenterally-administered conjugate vaccine against type B *Haemophilus influenza* resulted in a reduction of carriage and a reduced risk of horizontal transmission. This was hypothesized to be due to such IgG (60, 61).

Circulating *M. hyopneumoniae*-specific $TNF^{+}IFN\text{-}\gamma^{+}$ CD4 and CD8 T cells were identified in particular in the SWE_TLR,

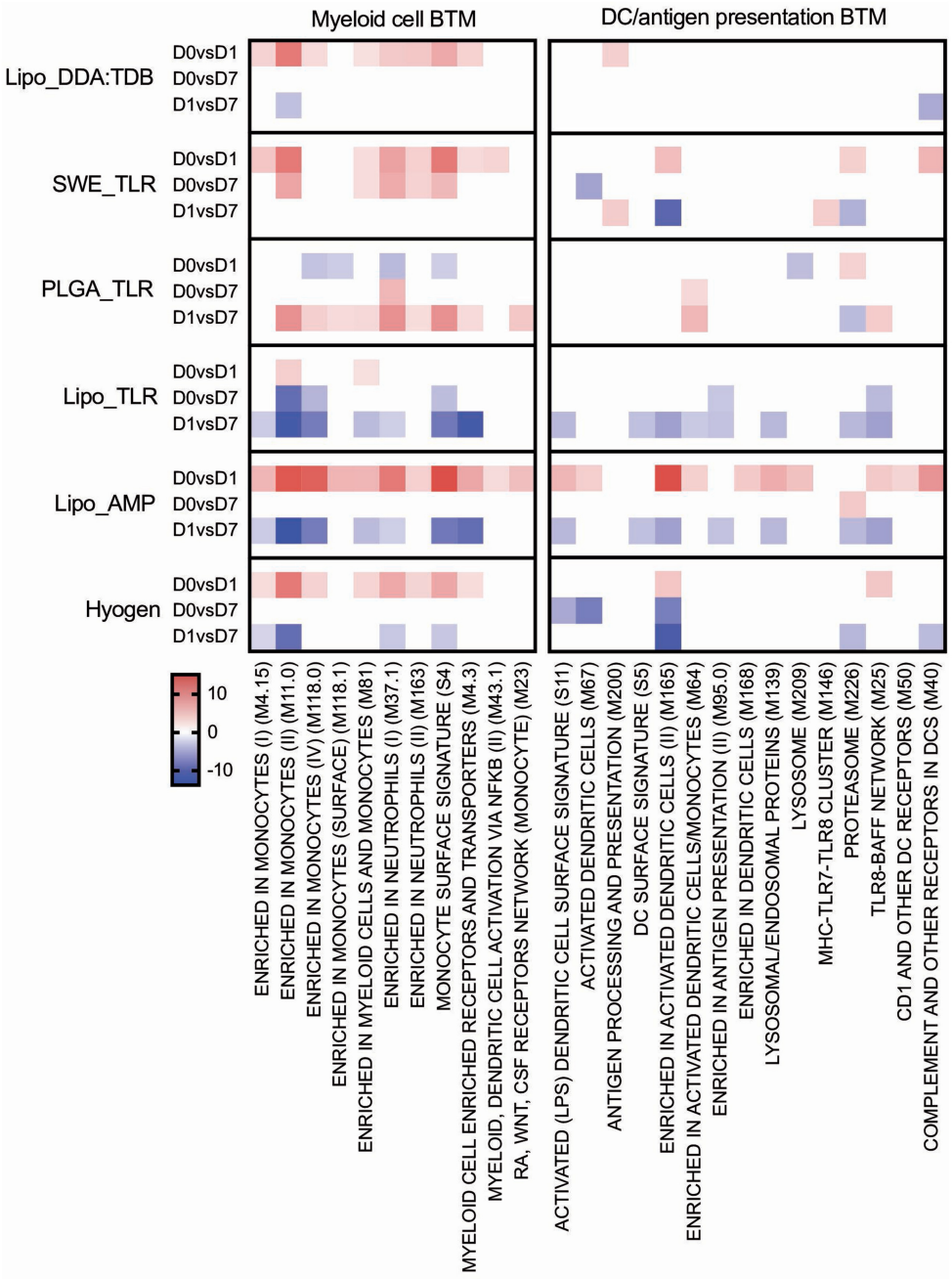

FIGURE 6 | Myeloid cell and DC/antigen presentation BTM induced by vaccines. The heat maps show the vaccine-dependent induction of BTM activity determined for D0 to D1 (D0 vs. D1), for D0 to D7 (D0 vs. D7), and for D1 to D7 (D1 vs. D7) changes in the modules. The values shown were calculated by −log(P-value)*1 for positively enriched BTM and as −log(P-value)* −1 for negatively enriched BTM. A cut-off of an FDR of $q < 0.1$ was employed. Red colors indicate BTM upregulation and blue downregulation. BTM, blood transcriptional modules.

Lipo_DDA:TDB, and Lipo_AMP groups. However, also in the Lipo_TLR and Hyogen groups a few animals appeared to have such cells. Such Th1 response is expected to promote cell-mediated immunity via activation of NK cells and macrophages, as well as by inducing antigen-specific cytotoxic immunity (CD8 cells) (62). While such responses could participate in protection against *M. hyopneumoniae,* pro-inflammatory CD4 Th responses might also mediate lung damage and clinical disease (63). While the classical effector functions of CD8 T cells are likely irrelevant for the immune response against a *Mycoplasma* species that is not an intracellular organism, mouse models indicate that CD8 T cells are suspected to dampen inflammatory responses mediated by $CD4^+$ Th cells (24, 64). Furthermore, CD8 T cells contribute to Th1 responses, which based on mouse models could

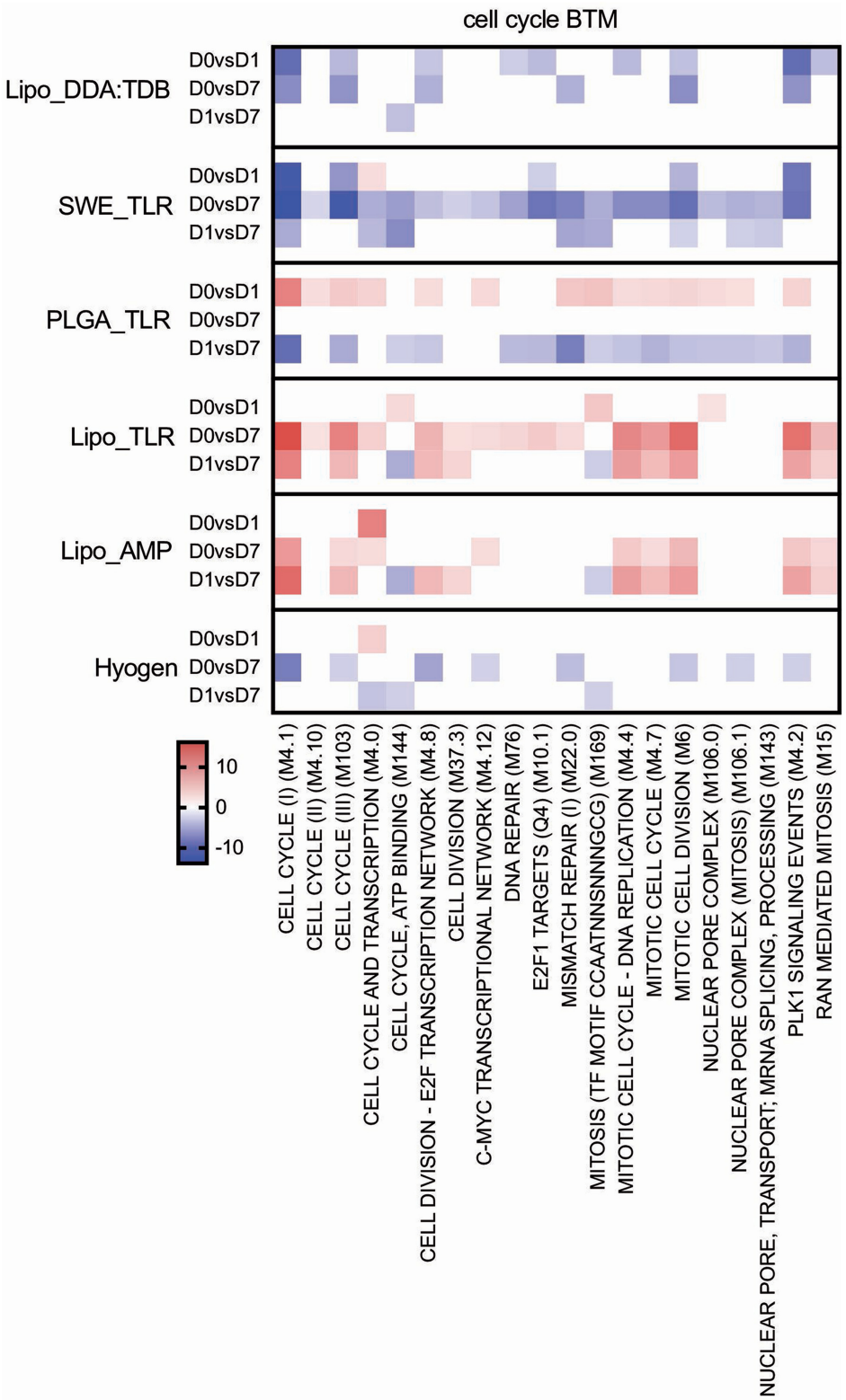

FIGURE 7 | Cell cycle BTM induced by vaccines. The heat maps show the vaccine-dependent induction of BTM activity determined for D0 to D1 (D0 vs. D1), for D0 to D7 (D0 vs. D7), and for D1 to D7 (D1 vs. D7) changes in the modules. The values shown were calculated by −log(P-value)*1 for positively enriched BTM and as −log(P-value)* −1 for negatively enriched BTM. A cut-off of an FDR of $q < 0.1$ was employed. Red colors indicate BTM upregulation and blue downregulation. BTM, blood transcriptional modules.

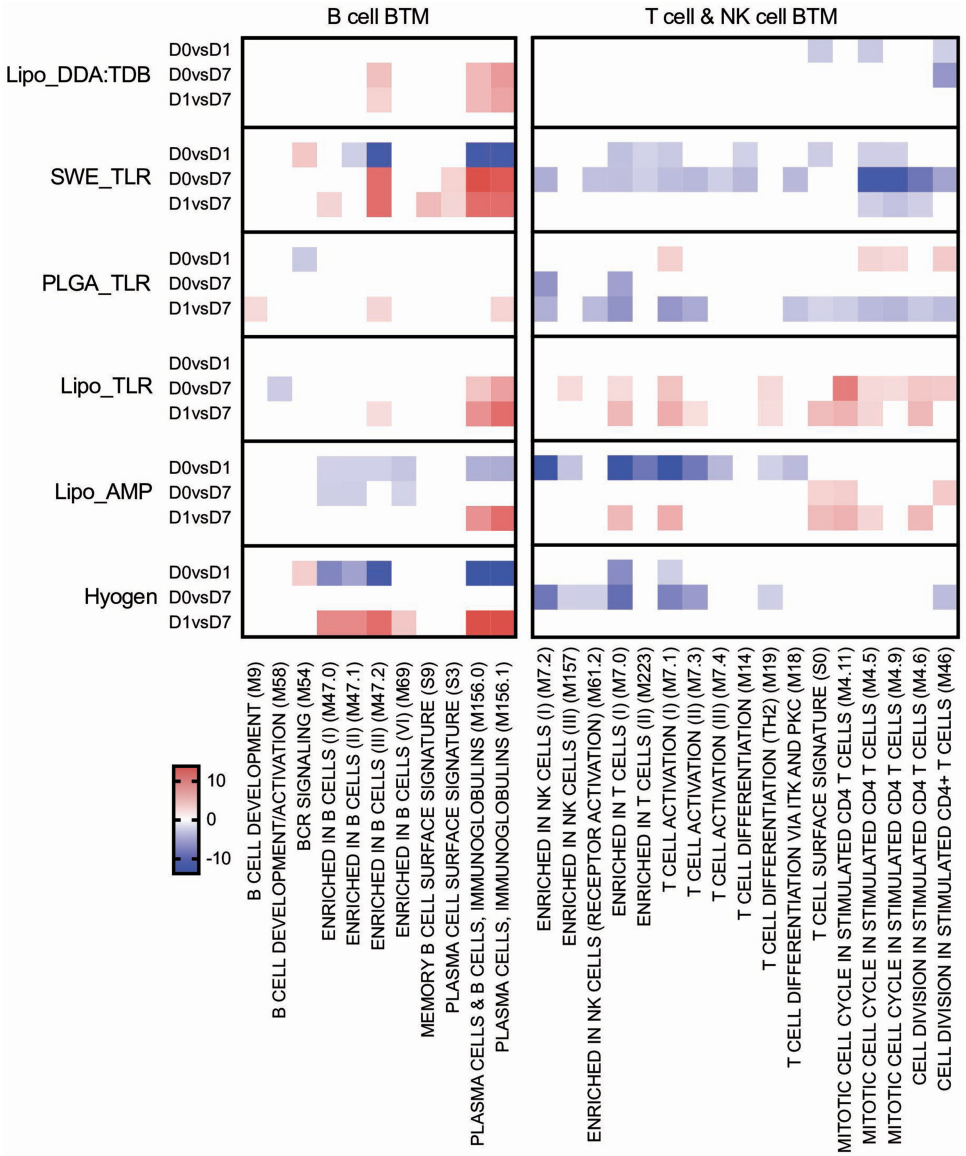

FIGURE 8 | B cell and T/NK cell BTM induced by vaccines. The heat maps show the vaccine-dependent induction of BTM activity determined for D0 to D1 (D0 vs. D1), for D0 to D7 (D0 vs. D7), and for D1 to D7 (D1 vs. D7) changes in the modules. The values shown were calculated by −log(P-value)*1 for positively enriched BTM and as −log(P-value)* −1 for negatively enriched BTM. A cut-off of an FDR of $q < 0.1$ was employed. Red colors indicate BTM upregulation and blue downregulation. BTM, blood transcriptional modules.

be protective against *Mycoplasma* infection. Based on this their induction by the present vaccines could be viewed as positive.

In this study, the PLGA_TLR formulation was the best at inducing a Th17 response, although in only three of six animals a response was detected. The lack of detection of IL-17-producing Th cells does not mean a lack of priming, as activated/memory T cells could have left the blood circulation. Nevertheless, future studies are required to confirm the ability of this formulation to induce Th17 responses. It has been suggested that Th17 cells may play a major role in the protection of the lung mucosa against respiratory pathogens by recruiting other immune cells to the inflamed mucosa for pathogen clearance (65) and by promoting IgA secretion into the airway lumen (66). Similar to other species, porcine IL-17-producing cell differentiation can be induced *in vitro* by TGF-β in the presence of IL-6 and/or IL-1β (67), and *in vivo* during several extracellular bacterial infections (68–70).

In addition to these classical vaccinology readouts we also applied a transcriptomics-based approach to obtain a more precise profile of the type of immune response induced by our vaccine candidates, and to better understand the immune modulatory effector functions needed for induction of a protective immune response after vaccination. We identified a number of BTM correlating to adaptive immune responses which have been previously reported in human and sheep

studies (25, 26, 28–30, 34). This was an early upregulation of monocytes BTM, such as S4, M11.0, M118.0, and M118.1, neutrophil BTM such as M163 and M37.1, modules related to inflammation and pathogen sensing such as M16, M146, and M37.0, and BTM related to antigen presentation such as M147, M139, and M209. Interestingly, many of these modules strongly negatively correlated with the antibody and CD8 T cell responses from D1 to D7. This suggests that a strong innate immune response in the first 24 h followed immediately by a down-regulation is associated with the initiation of a stronger adaptive immune response. This inverse correlation was also seen at later time points in a previous study using sheep (34). Similar to previous reports (25, 28, 29, 34) a few cell cycle, B cell, and T/NK cell BTM upregulated in the first 24 h after vaccination negatively correlated with adaptive immune responses. While these correlations were mainly found for the antibody responses, most of the modules correlating to CD4 T cell responses were found to be cell cycle BTM upregulated between D0 and D7. This was also reported by Qi et al. (30) who found a strong association of cell cycle and DNA repair BTM to virus-specific T cells (common BTM are M4.4, M4.12, M103, M76, M22.0, M22.1). The upregulation of T/NK cell modules between D0 and D7 positively correlated to CD8 T cell responses, as well as to the antibody responses between D1 and D7. Altogether, these results confirm the importance of early innate immune responses in the myeloid and DC cell compartment within the first 24 h for a potent vaccine-induced adaptive immune response. Clearly, the upregulation of myeloid cell and DC/antigen presentation BTM could partially reflect changes in cell population, i.e., those that are caused by enhanced hematopoiesis following stimulation of the innate immune system (71). Nevertheless, in a previous study we were unable to identify a significant increase in the circulation of monocytes, indicating that the BTM changes reflect more than changes in cell populations (34). This study also demonstrates that the main upregulated BTM from D0 or D1 to D7 correlating to adaptive immune responses are cell cycle and T/NK cell BTM. This could reflect the first recirculation of activated T cells leaving the lymph nodes that drain the site of vaccine injection.

After obtaining this information, we went back and analyzed which BTM were actually induced by the vaccines. While all vaccines, with the exception of the PLGA_TLR formulation, induced early upregulation of inflammatory, myeloid cell and DC/antigen presentation BTM, the Lipo_AMP vaccine appeared to be the most potent in stimulating these early innate immune responses. When it came to the later upregulation of cell cycle and T/NK cell BTM, this was only a feature of the Lipo_AMP and the Lipo_TLR vaccines. This was surprising considering that the Lipo_TLR was not found to be a particularly potent formulation. Furthermore, the more potent vaccines, such as Lipo_DDA:TDB and SWE_TLR, actually induced a downregulation of these BTM. While this requires further investigations, our current interpretation is that there could be differences in the kinetics of activated lymphocyte recirculation, which would have required more frequent sampling to detect. Furthermore, it should again be noted that T cell recirculation and the presence of memory T cells in the circulation is a dynamic process. Therefore, a lack of antigen-specific T cells in the peripheral blood cannot be interpreted as a lack of priming. On the other hand, the BTM profile induced by the PLGA_TLR vaccine was in line with its rather poor immunogenicity.

Overall, our data demonstrate the potency of cationic liposome formulations as delivery system to induce potent B and T cell responses using inactivated *M. hyopneumoniae* as antigen. Cationic liposomes may have the advantage of a more targeted delivery of the immunostimulant and antigen to DC, and also have been shown to enhance the retention time in lymph nodes (72, 73). This may favor strong T cell responses. Although we did not specifically address the requirement of an immunostimulant for liposomal vaccines, it is well-described that immunogenicity of liposomal vaccines can be enhanced (72). Our data indicate that both AMP and TDB appear to be good candidate molecules for the *M. hyopneumoniae* vaccine. The transcriptomic profile of the Lipo_AMP vaccine was particularly impressive as it corresponded best to a BTM profile known to correlate with adaptive immune responses. From all experimental formulations, Lipo_DDA:TDB induced the highest antibody and Th1 responses. Unfortunately, this formulation caused a prolonged ISR after ID administration. Applying this vaccine only via the IM route could resolve this safety issue, but it would be probably associated with a loss of immunogenicity (74). In contrast, the PLGA-based MP formulation did not appear to be suitable to induce good antibody and Th1 responses, possibly in part due to a delayed TLR ligand delivery to innate immune cells. Nevertheless, the fact that this vaccine induced IL17-producing Th cells at least in some animals is interesting and should be kept in mind for future investigations. The present work also identified the SWE_TLR as an interesting vaccine candidate, as it induced a robust Th1 response and IgA in BAL fluid of one animal. This vaccine has the advantage of being easy to produce. Moreover, O/W formulations are known to have a much better safety profile as W/O vaccines (72). Future studies are required to address which immunostimulant is best suited for a SWE adjuvant in the pig. This will require the use of a selection of identical antigens.

In conclusion, the present study identified promising *M. hyopneumoniae* bacterin formulations to be selected for future challenge experiments, based on their ability to induce strong innate immune responses and robust Th1 or Th17 responses. We also demonstrated the utility of transcriptome-based systems immunology analyses to unravel the mechanistic events leading to the stimulation of adaptive immune responses after vaccine injection. While the present study was not designed to identify the effects of formulation and immunostimulants but rather to select the most promising candidates from five novel vaccines, the information provided on these vaccine formulations will also be very valuable for other vaccines and future adjuvant research.

ETHICS STATEMENT

This study was carried out in accordance with the following laws and directives on animal experimentation in Belgium: 'KB 29/05/2013 betreffende de bescherming van proefdieren, KB 31/12/2012 herziening wet 86, ETS 123 richtlijnen huisvesting

proefdieren' and the EU 2010/63 directive on the protection of animals used for scientific purposes. The protocol was approved by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (approval number EC2016/91).

AUTHOR CONTRIBUTIONS

AMM, GA, OG-N, ROB, BD, and AM performed the animal experimentation, acquisition and analyses of data. VJ, CG, CB-Q, and NC designed, produced, and characterized the vaccines. IK, RB, and AS performed the bioinformatic analyses. DM, CB-Q, FB, FH, and AS designed and supervised the overall project.

FUNDING

This study was financially supported by the European H2020 Project SAPHIR (Project No. 633184). BD is supported by a postdoctoral grant of the Research Foundation—Flanders (FWO-Vlaanderen).

ACKNOWLEDGMENTS

We are grateful to Muriel Fragnière and Tosso Leeb from the Next Generation Sequencing Platform of the University of Bern, and Corinne Hug from the Institute of Virology and Immunology for the RNA preparation and sequencing. We would like to thank as well Dr. Ilias Chantziaras from the Porcine Health Management Unit of the University of Ghent for his statistical advice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01087/full#supplementary-material

Data Sheet 1 | List of genes for each BTM.

REFERENCES

1. Rautiainen E, Virtala AM, Wallgren P, Saloniemi H. Varying effects of infections with *Mycoplasma hyopneumoniae* on the weight gain recorded in three different multisource fattening pig herds. *J Vet Med Ser B*. (2000) 47:461–9. doi: 10.1046/j.1439-0450.2000.00370.x
2. Maes D, Segales J, Meyns T, Sibila M, Pieters M, Haesebrouck F. Control of *Mycoplasma hyopneumoniae* infections in pigs. *Vet Microbiol*. (2008) 126:297–309. doi: 10.1016/j.vetmic.2007.09.008
3. Martínez J, Peris B, Gómez EA, Corpa JM. The relationship between infectious and non-infectious herd factors with pneumonia at slaughter and productive parameters in fattening pigs. *Vet J*. (2009) 179:240–6. doi: 10.1016/j.tvjl.2007.10.006
4. Maes D, Sibila M, Kuhnert P, Segalés J, Haesebrouck F, Pieters M. Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. *Transbound Emerg Dis*. (2018) 65:110–24. doi: 10.1111/tbed.12677
5. Thacker EL, Thacker BJ, Boettcher TB, Jayappa H. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. *J Swine Health Prod*. (1998) 6:107–12.
6. Meyns T, Dewulf J, De Kruif A, Calus D, Haesebrouck F, Maes D. Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. *Vaccine*. (2006) 24:7081–6. doi: 10.1016/j.vaccine.2006.07.004
7. Villarreal I, Meyns T, Dewulf J, Vranckx K, Calus D, Pasmans F, et al. The effect of vaccination on the transmission of *Mycoplasma hyopneumoniae* in pigs under field conditions. *Vet J*. (2011) 188:48–52. doi: 10.1016/j.tvjl.2010.04.024
8. Goodwin R, Whittlestone P. Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. *Br J Exp Pathol*. (1963) 44:291.
9. Stakenborg T, Vicca J, Butaye P, Maes D, Peeters J, De Kruif A, et al. The diversity of *Mycoplasma hyopneumoniae* within and between herds using pulsed-field gel electrophoresis. *Vet Microbiol*. (2005) 109:29–36. doi: 10.1016/j.vetmic.2005.05.005
10. Villarreal I, Vranckx K, Calus D, Pasmans F, Haesebrouck F, Maes D. Effect of challenge of pigs previously immunised with inactivated vaccines containing homologous and heterologous *Mycoplasma hyopneumoniae* strains. *BMC Vet Res*. (2012) 8:2. doi: 10.1186/1746-6148-8-2
11. Jensen C, Ersbøll AK, Nielsen JP. A meta-analysis comparing the effect of vaccines against *Mycoplasma hyopneumoniae* on daily weight gain in pigs. *Prev Vet Med*. (2002) 54:265–78. doi: 10.1016/S0167-5877(02)00005-3
12. Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A. Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Vet Microbiol*. (2004) 100:255–68. doi: 10.1016/j.vetmic.2004.03.002
13. Djordjevic S, Eamens G, Romalis L, Nicholls P, Taylor V, Chin J. Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. *Austr Veter J*. (1997) 75:504–11. doi: 10.1111/j.1751-0813.1997.tb14383.x
14. Thacker EL, Thacker BJ, Kuhn M, Hawkins PA, Waters WR. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. *Am J Vet Res*. (2000) 61:1384–9. doi: 10.2460/ajvr.2000.61.1384
15. Marchioro SB, Maes D, Flahou B, Pasmans F, Sacristán RDP, Vranckx K, et al. Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae vaccine*. *Vaccine*. (2013) 31:1305–11. doi: 10.1016/j.vaccine.2012.12.068
16. Seo HW, Han K, Oh Y, Park C, Choo EJ, Kim S-H, et al. Comparison of cell-mediated immunity induced by three commercial single-dose *Mycoplasma hyopneumoniae* bacterins in pigs. *J Vet Med Sci*. (2013) 75:245–7. doi: 10.1292/jvms.12-0292
17. Martelli P, Saleri R, Cavalli V, De Angelis E, Ferrari L, Benetti M, et al. Systemic and local immune response in pigs intradermally and intramuscularly injected with inactivated *Mycoplasma hyopneumoniae* vaccines. *Vet Microbiol*. (2014) 168:357–64. doi: 10.1016/j.vetmic.2013.11.025
18. Auray G, Keller I, Python S, Gerber M, Bruggmann R, Ruggli N, et al. Characterization and transcriptomic analysis of porcine blood conventional and plasmacytoid dendritic cells reveals striking species-specific differences. *J Immunol*. (2016) 197:4791–806. doi: 10.4049/jimmunol.1600672
19. Braun RO, Python S, Summerfield A. Porcine B cell subset responses to toll-like receptor ligands. *Front Immunol*. (2017) 8:1044. doi: 10.3389/fimmu.2017.01044
20. Libanova R, Becker PD, Guzmán CA. Cyclic di-nucleotides: new era for small molecules as adjuvants. *Microb Biotechnol*. (2012) 5:168–76. doi: 10.1111/j.1751-7915.2011.00306.x
21. Tandrup Schmidt S, Foged C, Korsholm KS, Rades T, Christensen D. Liposome-based adjuvants for subunit vaccines: formulation strategies for subunit antigens and immunostimulators. *Pharmaceutics*. (2016) 8:E7. doi: 10.3390/pharmaceutics8010007
22. Vicca J, Stakenborg T, Maes D, Butaye P, Peeters J, De Kruif A, et al. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. *Vet Microbiol*. (2003) 97:177–90. doi: 10.1016/j.vetmic.2003.08.008

23. Calus D, Baele M, Meyns T, De Kruif A, Butaye P, Decostere A, et al. Protein variability among *Mycoplasma hyopneumoniae* isolates. *Vet Microb*. (2007) 120:284–91. doi: 10.1016/j.vetmic.2006.10.040
24. Dobbs NA, Odeh AN, Sun X, Simecka JW. The multifaceted role of T cell-mediated immunity in pathogenesis and resistance to *Mycoplasma* respiratory disease. *Curr Trends Immunol.* (2009) 10:1–19.
25. Li S, Rouphael N, Duraisingham S, Romero-Steiner S, Presnell S, Davis C, et al. Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. *Nat Immunol*. (2014) 15:195–204. doi: 10.1038/ni.2789
26. Matsumiya M, Harris SA, Satti I, Stockdale L, Tanner R, O'shea MK, et al. Inflammatory and myeloid-associated gene expression before and one day after infant vaccination with MVA85A correlates with induction of a T cell response. *BMC Infect Dis*. (2014) 14:314. doi: 10.1186/1471-2334-14-314
27. Hagan T, Nakaya HI, Subramaniam S, Pulendran B. Systems vaccinology: enabling rational vaccine design with systems biological approaches. *Vaccine*. (2015) 33:5294–301. doi: 10.1016/j.vaccine.2015.03.072
28. Nakaya HI, Hagan T, Duraisingham SS, Lee EK, Kwissa M, Rouphael N, et al. Systems analysis of *Immunity* to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures. *Immunity*. (2015) 43:1186–98. doi: 10.1016/j.immuni.2015.11.012
29. Nakaya HI, Clutterbuck E, Kazmin D, Wang L, Cortese M, Bosinger SE, et al. Systems biology of immunity to MF59-adjuvanted versus nonadjuvanted trivalent seasonal influenza vaccines in early childhood. *Proc Natl Acad Sci USA*. (2016) 113:1853–8. doi: 10.1073/pnas.1519690113
30. Qi Q, Cavanagh MM, Le Saux S, Wagar LE, Mackey S, Hu J, et al. Defective T memory cell differentiation after varicella zoster vaccination in older individuals. *PLoS Pathog*. (2016) 12:e1005892. doi: 10.1371/journal.ppat.1005892
31. Hou J, Wang S, Jia M, Li D, Liu Y, Li Z, et al. A systems vaccinology approach reveals temporal transcriptomic changes of immune responses to the yellow fever 17D vaccine. *J Immunol*. (2017) 199:1476–89. doi: 10.4049/jimmunol.1700083
32. Kazmin D, Nakaya HI, Lee EK, Johnson MJ, Van Der Most R, Van Den Berg RA, et al. Systems analysis of protective immune responses to RTS,S malaria vaccination in humans. *Proc Natl Acad Sci USA*. (2017) 114:2425–30. doi: 10.1073/pnas.1621489114
33. Li S, Sullivan NL, Rouphael N, Yu T, Banton S, Maddur MS, et al. Metabolic phenotypes of response to vaccination in humans. *Cell*. (2017) 169:862–877.e17. doi: 10.1016/j.cell.2017.04.026
34. Braun RO, Brunner L, Wyler K, Auray G, Garcia-Nicolas O, Python S, et al. System immunology-based identification of blood transcriptional modules correlating to antibody responses in sheep. *NPJ Vaccines*. (2018) 3:41. doi: 10.1038/s41541-018-0078-0
35. Calus D, Maes D, Vranckx K, Villareal I, Pasmans F, Haesebrouck F. Validation of ATP luminometry for rapid and accurate titration of *Mycoplasma hyopneumoniae* in Friis medium and a comparison with the color changing units assay. *J Microbiol Methods*. (2010) 83:335–40. doi: 10.1016/j.mimet.2010.09.001
36. Christensen D, Foged C, Rosenkrands I, Nielsen HM, Andersen P, Agger EM. Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. *Biochim Biophys Acta Biomembr*. (2007) 1768:2120–9. doi: 10.1016/j.bbamem.2007.05.009
37. Barnier-Quer C, Elsharkawy A, Romeijn S, Kros A, Jiskoot W. Adjuvant effect of cationic liposomes for subunit influenza vaccine: influence of antigen loading method, cholesterol and immune modulators. *Pharmaceutics*. (2013) 5:392–410. doi: 10.3390/pharmaceutics5030392
38. Singh M, Briones M, Ott G, O'hagan D. Cationic microparticles: a potent delivery system for DNA vaccines. *Proc Natl Acad Sci*. (2000) 97:811–6. doi: 10.1073/pnas.97.2.811
39. Ventura R, Brunner L, Heriyanto B, De Boer O, O'hara M, Huynh C, et al. Technology transfer of an oil-in-water vaccine-adjuvant for strengthening pandemic influenza preparedness in Indonesia. *Vaccine*. (2013) 31:1641–5. doi: 10.1016/j.vaccine.2012.07.074
40. Dietrich J, Andreasen LV, Andersen P, Agger EM. Inducing dose sparing with inactivated polio virus formulated in adjuvant CAF01. *PLoS ONE*. (2014) 9:e100879. doi: 10.1371/journal.pone.0100879
41. Hannan P, Bhogal B, Fish J. Tylosin tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses. *Res Vet Sci*. (1982) 33:76–88. doi: 10.1016/S0034-5288(18)32364-6
42. Stärk KD, Nicolet J, Frey J. Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay. *Appl Environ Microbiol*. (1998) 64:543–8.
43. Sacristán RDP, Sierens A, Marchioro S, Vangroenweghe F, Jourquin J, Labarque G, et al. Efficacy of early *Mycoplasma hyopneumoniae* vaccination against mixed respiratory disease in older fattening pigs. *Vet Rec*. (2014) 174:197. doi: 10.1136/vr.101597
44. Bereiter M, Young T, Joo H, Ross R. Evaluation of the ELISA and comparison to the complement fixation test and radial immunodiffusion enzyme assay for detection of antibodies against *Mycoplasma hyopneumoniae* in swine serum. *Vet Microbiol*. (1990) 25:177–92. doi: 10.1016/0378-1135(90)90075-7
45. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. (2015) 12:357. doi: 10.1038/nmeth.3317
46. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. (2014) 15:550. doi: 10.1186/s13059-014-0550-8
47. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
48. Li S, Nakaya HI, Kazmin DA, Oh JZ, Pulendran B. Systems biological approaches to measure and understand vaccine immunity in humans. *Semin Immunol*. (2013) 25:209–18. doi: 10.1016/j.smim.2013.05.003
49. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*. (2009) 462:108–12. doi: 10.1038/nature08460
50. Llopart D, Casal J, Clota J, Navarra I, March R, Riera P, et al. Evaluation of the field efficacy and safety of a *Mycoplasma hyopneumoniae* vaccine in finishing pigs. *PIG J*. (2002) 49:70–83.
51. Roth JA. Mechanistic bases for adverse vaccine reactions and vaccine failures. *Adv Vet Med*. (1999) 41:681–700. doi: 10.1016/S0065-3519(99)80053-6
52. Meyer EK. Vaccine-associated adverse events. *Vet Clin Small Anim Pract*. (2001) 31:493–514. doi: 10.1016/S0195-5616(01)50604-X
53. Jones GF, Rapp-Gabrielson V, Wilke R, Thacker EL, Thacker BJ, Gergen L, et al. Intradermal vaccination for *Mycoplasma hyopneumoniae*. *J Swine Health Product*. (2005) 13:19–27.
54. Galliher-Beckley A, Pappan L, Madera R, Burakova Y, Waters A, Nickles M, et al. Characterization of a novel oil-in-water emulsion adjuvant for swine influenza virus and *Mycoplasma hyopneumoniae* vaccines. *Vaccine*. (2015) 33:2903–8. doi: 10.1016/j.vaccine.2015.04.065
55. Beffort L, Weiß C, Fiebig K, Jolie R, Ritzmann M, Eddicks M. Field study on the safety and efficacy of intradermal versus intramuscular vaccination against *Mycoplasma hyopneumoniae*. *Vet Rec*. (2017) 181:348. doi: 10.1136/vr.104466
56. Spickler AR, Roth JA. Adjuvants in veterinary vaccines: modes of action and adverse effects. *J Vet Int Med*. (2003) 17:273–81. doi: 10.1111/j.1939-1676.2003.tb02448.x
57. Xiong Q, Wei Y, Xie H, Feng Z, Gan Y, Wang C, et al. Effect of different adjuvant formulations on the immunogenicity and protective effect of a live *Mycoplasma hyopneumoniae* vaccine after intramuscular inoculation. *Vaccine*. (2014) 32:3445–51. doi: 10.1016/j.vaccine.2014.03.071
58. Binjawadagi B, Dwivedi V, Manickam C, Ouyang K, Wu Y, Lee LJ, et al. Adjuvanted poly(lactic-co-glycolic) acid nanoparticle-entrapped inactivated porcine reproductive and respiratory syndrome virus vaccine elicits cross-protective immune response in pigs. *Int J Nanomedicine*. (2014) 9:679–94. doi: 10.2147/IJN.S56127
59. Dhakal S, Renu S, Ghimire S, Shaan Lakshmanappa Y, Hogshead BT, Feliciano-Ruiz N, et al. Mucosal immunity and protective efficacy of intranasal inactivated influenza vaccine is improved by chitosan nanoparticle delivery in pigs. *Front Immunol*. (2018) 9:934. doi: 10.3389/fimmu.2018.00934
60. Kauppi M, Eskola J, Käyhty H. Anti-capsular polysaccharide antibody concentrations in saliva after immunization with *Haemophilus influenzae* type b conjugate vaccines. *Pediatr Infect Dis J*. (1995) 14:286–94. doi: 10.1097/00006454-199504000-00008
61. Wenger JD. Epidemiology of *Haemophilus influenzae* type b disease and impact of *Haemophilus influenzae* type b conjugate vaccines in

the United States and Canada. *Pediatr Infect Dis J*. (1998) 17:S132–6. doi: 10.1097/00006454-199809001-00008
62. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. *J Leukocyte Biol*. (2004) 75:163–89. doi: 10.1189/jlb.0603252
63. Jones HP, Simecka JW. T lymphocyte responses are critical determinants in the pathogenesis and resistance to *Mycoplasma* respiratory disease. *Front Biosci*. (2003) 8:930–45. doi: 10.2741/1098
64. Jones HP, Tabor L, Sun X, Woolard MD, Simecka JW. Depletion of CD8+ T cells exacerbates CD4+ Th cell-associated inflammatory lesions during murine *Mycoplasma* respiratory disease. *J Immunol*. (2002) 168:3493–501. doi: 10.4049/jimmunol.168.7.3493
65. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, et al. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J Immunol*. (2007) 179:7791–9. doi: 10.4049/jimmunol.179.11.7791
66. Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. *J Immunol*. (2009) 182:4507–11. doi: 10.4049/jimmunol.0900237
67. Kiros TG, Van Kessel J, Babiuk LA, Gerdts V. Induction, regulation and physiological role of IL-17 secreting helper T-cells isolated from PBMC, thymus, and lung lymphocytes of young pigs. *Vet Immunol Immunopathol*. (2011) 144:448–54. doi: 10.1016/j.vetimm.2011.08.021
68. Luo Y, Van Nguyen U, De La Fe Rodriguez PY, Devriendt B, Cox E. $F4^+$ ETEC infection and oral immunization with F4 fimbriae elicits an IL-17-dominated immune response. *Vet Res*. (2015) 46:121. doi: 10.1186/s13567-015-0264-2
69. De Witte C, Devriendt B, Flahou B, Bosschem I, Ducatelle R, Smet A, et al. *Helicobacter suis* induces changes in gastric inflammation and acid secretion markers in pigs of different ages. *Vet Res*. (2017) 48:34. doi: 10.1186/s13567-017-0441-6
70. Mullebner A, Sassu EL, Ladinig A, Frombling J, Miller I, Ehling-Schulz M, et al. *Actinobacillus pleuropneumoniae* triggers IL-10 expression in tonsils to mediate colonisation and persistence of infection in pigs. *Vet Immunol Immunopathol*. (2018) 205:17–23. doi: 10.1016/j.vetimm.2018.10.008
71. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. *Immunity*. (2018) 49:595–613. doi: 10.1016/j.immuni.2018.10.005
72. Bonam SR, Partidos CD, Halmuthur SKM, Muller S. An overview of novel adjuvants designed for improving vaccine efficacy. *Trends Pharmacol Sci*. (2017) 38:771–93. doi: 10.1016/j.tips.2017.06.002
73. Bookstaver ML, Tsai SJ, Bromberg JS, Jewell CM. Improving vaccine and immunotherapy design using biomaterials. *Trends Immunol*. (2018) 39:135–50. doi: 10.1016/j.it.2017.10.002
74. Combadiere B, Liard C. Transcutaneous and intradermal vaccination. *Hum Vaccines*. (2011) 7:811–27. doi: 10.4161/hv.7.8.16274

Conflict of Interest Statement: CG is named as inventor in a patent covering the use of c-di-AMP as adjuvant (PCT/EP 2006010693).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Maes *et al. Vet Res* (2021) 52:67
https://doi.org/10.1186/s13567-021-00941-x

VETERINARY RESEARCH

REVIEW Open Access

Perspectives for improvement of *Mycoplasma hyopneumoniae* vaccines in pigs

Dominiek Maes[1*], Filip Boyen[1], Bert Devriendt[1], Peter Kuhnert[2], Artur Summerfield[3,4] and Freddy Haesebrouck[1]

Abstract

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is one of the primary agents involved in the porcine respiratory disease complex, economically one of the most important diseases in pigs worldwide. The pathogen adheres to the ciliated epithelium of the trachea, bronchi, and bronchioles, causes damage to the mucosal clearance system, modulates the immune system and renders the animal more susceptible to other respiratory infections. The pathogenesis is very complex and not yet fully understood. Cell-mediated and likely also mucosal humoral responses are considered important for protection, although infected animals are not able to rapidly clear the pathogen from the respiratory tract. Vaccination is frequently practiced worldwide to control *M. hyopneumoniae* infections and the associated performance losses, animal welfare issues, and treatment costs. Commercial vaccines are mostly bacterins that are administered intramuscularly. However, the commercial vaccines provide only partial protection, they do not prevent infection and have a limited effect on transmission. Therefore, there is a need for novel vaccines that confer a better protection. The present paper gives a short overview of the pathogenesis and immune responses following *M. hyopneumoniae* infection, outlines the major limitations of the commercial vaccines and reviews the different experimental *M. hyopneumoniae* vaccines that have been developed and tested in mice and pigs. Most experimental subunit, DNA and vector vaccines are based on the P97 adhesin or other factors that are important for pathogen survival and pathogenesis. Other studies focused on bacterins combined with novel adjuvants. Very few efforts have been directed towards the development of attenuated vaccines, although such vaccines may have great potential. As cell-mediated and likely also humoral mucosal responses are important for protection, new vaccines should aim to target these arms of the immune response. The selection of proper antigens, administration route and type of adjuvant and carrier molecule is essential for success. Also practical aspects, such as cost of the vaccine, ease of production, transport and administration, and possible combination with vaccines against other porcine pathogens, are important. Possible avenues for further research to develop better vaccines and to achieve a more sustainable control of *M. hyopneumoniae* infections are discussed.

Keywords: *Mycoplasma hyopneumoniae*, Vaccination, Immune responses, Experimental vaccines, Pig

Table of Contents

*Correspondence: Dominiek.Maes@UGent.be
[1] Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
Full list of author information is available at the end of the article

1 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the most important *Mycoplasma* sp. in swine health management. It is the primary pathogen of enzootic pneumonia, a chronic respiratory disease in pigs, and one of the primary agents involved in the porcine respiratory disease complex [1]. Infections with *M. hyopneumoniae* are highly prevalent in almost all swine producing areas, and they cause significant economic losses due to increased medication use and decreased performance of the pigs [2].

Similar to other mycoplasmas, *M. hyopneumoniae* lacks a cell wall. The organism is very difficult to isolate because of its slow growth and potential overgrowth with other swine mycoplasmas. The pathogen-host interactions are very complex and not fully characterized. The organism is primarily identified on the mucosal surface of the trachea, bronchi, and bronchioles [3]. It affects the mucosal clearance system by disrupting the cilia on the epithelial surface and, additionally, the organism modulates the immune system of the respiratory tract [4]. Therefore, *M. hyopneumoniae* predisposes animals to concurrent infections with other respiratory pathogens.

Control of *M. hyopneumoniae* infections in pig herds can be accomplished by optimizing management, housing and biosecurity practices [5]. Treatment can be done using medication with antimicrobials active against *M. hyopneumoniae*. Antimicrobial medication can limit the consequences of the disease and decrease the infection load [6], but it does not prevent pigs from becoming infected with *M. hyopneumoniae*. Medication with antimicrobials is also discouraged because of the risk of antimicrobial resistance development [7]. Vaccination against *M. hyopneumoniae* has been shown to be a useful tool to control *M. hyopneumoniae* infections. Different inactivated, whole-cell vaccines are commercially available and vaccination is frequently practiced worldwide. In infected herds, vaccination decreases clinical signs and lung lesions due to *M. hyopneumoniae* infections, performance losses of the animals and antimicrobial use. Although the commercial vaccines are widely used and cost-efficient in many farms, they induce only a partial protection and do not prevent infection.

The present paper discusses the complex interaction of *M. hyopneumoniae* with the host and the limitations of the currently available commercial vaccines. Next, the different experimental vaccines that have been developed and tested in mice and pigs are reviewed. Finally, avenues for further research are provided in order to improve *M. hyopneumoniae* vaccination and achieve a more sustainable control of *M. hyopneumoniae* infections.

2 Interactions of *M. hyopneumoniae* with the host

2.1 The organism

M. hyopneumoniae is a small (0.2–0.4 µm) and pleomorphic organism as it lacks a shape-defining cell-wall. Studies have shown a high diversity at genomic, antigenic, and proteomic level between different strains [8]. The genomes of the earliest described *M. hyopneumoniae* strains (the pathogenic strains 232 and 7448, and the nonpathogenic strain J) were sequenced in 2004 [9, 10]. Since then, the genomes of several other strains have been sequenced. Currently, 23 entirely sequenced *M. hyopneumoniae* genomes are available, 11 already assembled and annotated, and 12 not fully assembled. In general, the genomes are small in size, namely 0.86–0.96 Mb, and in each of them, there are 528 to 691 protein-encoding genes [11]. Despite the small genome, up to 30% of the gene content is still of unknown function [12]. In addition, 20 to 30% of the *M. hyopneumoniae* genes encode surface proteins, the function of many of them is not yet known [12]. The mean GC content, which influences genome organization and gene expression, is low (28.54%) compared to other bacterial species. The low GC content gives *M. hyopneumoniae* a complex transcriptional organization, unique intrinsic terminator stem-loop formation and individual ribonuclease P (RNase P) structure [13].

The small genome of *M. hyopneumoniae* and the limited number of secreted or surface proteins is particularly interesting for use of recombinant DNA technology. However, *M. hyopneumoniae* uses an unusual genetic code. The amino acid tryptophan is not encoded by TGG, but by TGA, which is a stop codon in most organisms [14]. This difference has hampered the expression of genes of *M. hyopneumoniae* containing TGA codons in *E. coli*, the most attractive system used for production of recombinant proteins. However, mutations replacing TGA codons with TGG have been used successfully to solve this problem [15].

2.2 Adherence

Upon inhalation, *M. hyopneumoniae* adheres to the ciliated epithelial cells of the trachea, bronchi and bronchioles underneath the mucous layer. Adhesion is followed by the induction of ciliostasis, loss of cilia, and eventually epithelial cell death [16].

A repertoire of at least 35 M. *hyopneumoniae* proteins have been associated with cell adhesion, including several related to the P97/P102 paralog families and other surface proteins that moonlight as adhesins [17, 18]. The number of *M. hyopneumoniae* adhesins can even be higher, considering that its surfaceome includes more than 290 proteins and that many uncharacterized surface displayed proteins may bear adhesion properties. Different adhesins may vary in abundance at the cell surface between strains. This may be due to differential transcriptional rates of the respective genes, differential translational rates of the corresponding mRNAs and/or post-translational events, including their export to the cell membrane and proteolytic processing. Proteolytic processing of adhesins can shape the bacterial surface architecture [19], generating several adhesin proteoforms, that may exert alternative functions. Some of them are displayed at the cell surface, while others may stay in the cytoplasm or be released from the cell membrane to the extracellular milieu [11].

P97 is one of the most important adhesins of *M. hyopneumoniae* and also the most intensively studied one, and therefore, it has been tested in many experimental vaccines. P97 contains two repeat regions (R1 and R2), located in the C-terminal portion. The sites that are involved in cilium binding are located in the R1 region and at least seven AAKPV/E repeats are required for functional binding. Both R1 and R2 are involved in the attachment of *M. hyopneumoniae* to the extracellular matrix of the respiratory tract [20].

Apart from the cilia-exposed glycans, some swine extracellular matrix molecules, such as fibronectin and plasminogen, also provide binding sites for surface adhesins of *M. hyopneumoniae* [11]. The fibronectin- and plasminogen-binding ability of *M. hyopneumoniae* may mediate subsequent adherence to swine respiratory cilia. Further research is needed to investigate whether adherence to fibronectin and plasminogen may facilitate internalization of *M. hyopneumoniae* and facilitate its traffic via the circulatory system and penetration into host organs, such as liver, kidneys and spleen [21]. The exact role of plasmin in the chronic inflammatory response as observed during *M. hyopneumoniae* infection is unclear, but it may influence the migration of inflammatory cells and stimulate the release of pro-inflammatory cytokines [22].

Extracellular actin is also used as a surface receptor by different proteoforms of *M. hyopneumoniae* P97 adhesin and other proteins, including lipoproteins, glycolytic enzymes, chaperones and translation factors [23]. Apart from extracellular actin, surface proteins of *M. hyopneumoniae* also interact with other cytoskeletal proteins, such as vimentin, keratin, tubulin, myosin, and tropomyosin [24].

2.3 Virulence factors

Adhesion serves as the starting point of infection which is then assisted by other virulence factors. Classical virulence factors like toxins are generally lacking in *Mycoplasma* species. *Mycoplasma hyopneumoniae* can produce H_2O_2 in the presence of glycerol in vitro. However, this is strain dependent and the attenuated type strain J does not produce detectable amounts of H_2O_2 [25]. Whether production of hydrogen peroxide should be considered as a possible in vivo virulence mechanism in *M. hyopneumoniae* remains to be investigated. Ferrarini et al. [25] showed that *M. hyopneumoniae* is able to take up myo-inositol and use it as an alternative energy source in the absence of glucose. Since myo-inositol is freely available in the serum of pigs, it might be a suitable alternative energy source for *M. hyopneumoniae* residing in the highly vascularized lungs.

Lipid associated membrane proteins (LAMP) have also been implicated in the pathogenicity of mycoplasmas. They interact with the host immune system mainly through Toll-like receptors (TLRs), such as TLR2 [26]. In *M. hyopneumoniae*, whole membrane lipoprotein fractions induced apoptosis in various cell types, including porcine peripheral blood mononuclear cells (PBMCs) [27]. Furthermore, LAMPs activate production of nitric oxide and reactive oxygen species in the host cell [28].

Mycoplasmas need to scavenge nutrients including nucleotides from their environment and therefore, they are well known for their potent membrane nucleases [14]. A well-recognized membrane nuclease is MnuA. Macrophages and neutrophils may form extracellular traps (METs or NETs, respectively), consisting of an interlacement of chromatin fibres rich in DNA, host defense proteins and enzymes, allowing immobilization and killing of invading microbes. In *Mycoplasma bovis (M. bovis)*, MnuA was shown to degrade DNA-based neutrophil and macrophage extracellular traps (NETs and METs, respectively), thereby enabling *M. bovis* to escape these traps [29] and not being killed by these innate immune cells. The nuclease-encoding *mnuA* gene is also present in *M. hyopneumoniae* [30]. Therefore, MnuA could be a surface nuclease that is responsible for the degradation of NETs/METs, allowing *M. hyopneumoniae* to escape the host immune defense and using at the same time the

nucleotides and protein synthesis materials as nutrients for proliferation [31].

Mycoplasma hyopneumoniae may release extracellular DNA that allows the organism to form biofilms on abiotic and host surfaces [32]. Biofilm formation makes the pathogen more resistant to antimicrobials and the host immune responses. The molecular interactions and cellular processes underlying *M. hyopneumoniae* biofilm formation are thus far mostly unknown.

In *Mycoplasma genitalium*, an immunoglobulin (Ig) G binding protein, called protein M, has been identified. This protein not only fixes IgG very efficiently but also prevents subsequent antigen–antibody binding and subsequent signaling pathways of the bound antibodies [33]. In vitro experiments have shown that genes from *Mycoplasma mycoides* subspecies *capri* encode a *Mycoplasma* Ig binding protein (MIB) and a *Mycoplasma* Ig protease (MIP). The complex of MIB and Ig is necessary for the proteolytic activity of MIP. The two proteins are encoded by two genes and are often detected in multiple copies in various *Mycoplasma* sp., including *M. hyopneumoniae* [34]. Further studies are needed to investigate the role of the MIB-MIP system in virulence and immune evasion of *M. hyopneumoniae*.

Integrative and conjugative elements (ICE) are self-transmissible mobile genetic elements involved in horizontal gene transfer, thereby providing new virulence and/or antibiotic resistance traits. Such ICE have been identified in *M. hyopneumoniae* [35], but their role in encoding virulence traits of *M. hyopneumoniae* remains unclear.

Several large-scale comparisons on the genome, transcriptome, proteome, metabolome and secretome level have been performed in order to investigate virulence and pathogenesis of *M. hyopneumoniae* [24, 36]. Liu et al. reported that besides the known virulence-associated proteins (mainly adhesins), mutations were also found in genes involved in metabolism and growth [36]. *M. hyopneumoniae* is a genome-reduced organism that is characterized by a limited set of biosynthetic pathways, as such it is not surprising that further loss of enzymatic functions might have a large influence on survival and growth of the microorganism. This also holds true for lipoproteins involved in nutrient acquisition [37]. A comprehensive proteome profiling of two *M. hyopneumoniae* strains and *M. flocculare* provided tens of novel candidates to enzootic pneumonia determinants or virulence factors [24].

2.4 Immune responses

The interaction of the pathogen with the immune system of the host is not yet fully elucidated, and it is clear that some components of the immune system may both help and hinder the development of *Mycoplasma*-induced pneumonia [17, 38]. Infection induces the production of pro-inflammatory (e.g. interleukin (IL)-1β, IL-6, TNF-α) and immunoregulatory (e.g. IL-10) cytokines by macrophages, neutrophils and lymphocytes in the lung. This excessive inflammatory response is associated with lymphoid hyperplasia and is considered to be a major driver of lung lesions [39].

2.4.1 Innate immune responses

It has been shown that Toll-like receptor 2 (TLR2) and TLR6 are important in the recognition of *M. hyopneumoniae* by porcine alveolar macrophages [40]. The activation of this signal pathway leads to the production of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 by alveolar macrophages. Blocking TLR2 and TLR6 receptors led to less TNF-α production by macrophages [41], indicating that alveolar macrophages are involved in inflammatory and innate immune responses during *M. hyopneumoniae* infection. *M. hyopneumoniae* was also demonstrated to strongly activate monocytes and B cells in vitro, with the B cell-activation resulting in a potent polyreactive antibody response [42]. Nevertheless, the role of these responses in protection against *M. hyopneumoniae* infection is still unknown. Also, it is not clear why there is a low neutrophil infiltration upon *M. hyopneumoniae* infection.

Recently, Mucha et al. [43] investigated changes in gene expression of swine epithelial cells of the trachea upon infection with *M. hyopneumoniae*. Among the up-regulated genes, they found several genes related to immune response and inflammation, such as C3 complement, *SAA3*, chemokines (*CXCL2* and *CCL20*) and galectins. These chemokines may attract myeloid cells. The study also suggested that ciliostasis caused by this pathogen might partially be explained by the down-regulation of ciliary genes. The innate immune responses against *M. hyopneumoniae* and mycoplasma in general have been reviewed in more detail elsewhere [4].

2.4.2 Humoral responses

After experimental infection, *M. hyopneumoniae*-specific serum IgG antibodies are detected 3–4 weeks post-infection (pi), peak after 11–12 weeks and then decrease very gradually [44]. After booster infection, serum antibody titers clearly increase and then slowly decline again [44]. Interestingly, pigs infected with a highly virulent strain appear to seroconvert earlier than pigs infected with a low virulent strain [45]. *Mycoplasma hyopneumoniae*-specific IgM in serum can be detected as early as 9 days pi under experimental conditions. The percentage of IgM positive pigs peaks at 14 days pi and rapidly decreases afterwards [46]. When infection occurs naturally, seroconversion is

usually slower. Local *M. hyopneumoniae*-specific antibodies precede specific serum antibodies following infection but decline faster [47]. *Mycoplasma hyopneumoniae*-specific IgA can be detected in nasal swabs as early as 6 days pi, peak 12–16 days pi and decline steadily afterwards to reach pre-immune levels by day 84 pi.

Mycoplasma hyopneumoniae-specific IgG levels in serum induced by vaccination are not correlated with the severity of lung lesions in *M. hyopneumoniae*-infected pigs, suggesting that systemic antibodies play a minor role in protective immunity [48]. The role of mucosal antibodies in the protection against *M. hyopneumoniae* is still unclear. Some studies demonstrated that specific antibody levels in the respiratory tract did not correlate with protection [48, 49], whereas other studies emphasized the role of *M. hyopneumoniae*-specific secretory IgA in preventing adhesion of the microorganism to the ciliated cells of the respiratory tract [50–52]. Furthermore, specific IgG diffusing from the blood into the lung tissue or produced locally in the BALT could opsonize *M. hyopneumoniae*, resulting in phagocytosis by macrophages and neutrophils [53]. However, Deeney et al. [54] recently reported that addition of convalescent porcine sera did not enhance engulfment of *M. hyopneumoniae* by alveolar macrophages in vitro.

2.4.3 Cell-mediated responses

T cell-mediated immune responses are generally considered important for protection against Mycoplasmas causing local respiratory infections such as *M. hyopneumoniae* [4]. T cells are key in the regulation of immune responses and have a critical impact on the development of *Mycoplasma*-induced pneumonia [38]. *Mycoplasma pulmonis* challenge studies using various T cell subset-depleted mice indicate that T helper 1 (Th1), Th17 and $CD8^+$ T cell responses are responsible for protection against *Mycoplasma* disease. In contrast, Th2 responses are less efficient in controlling the infection and thus contribute to immunopathology [38]. In a *M. hyopneumoniae* vaccination-challenge study resulting in a significant reduction of lung lesions in the vaccinated group, Thacker et al. [50] observed a higher level of IFN-γ-secreting blood lymphocytes in vaccinated pigs compared to non-vaccinated ones before and after experimental infection. In *M. hyopneumoniae*-vaccination studies using a *M. hyopneumoniae*-resistant and a non-resistant pig line, higher serum levels of IFN-γ and IL-17A, but lower levels of IL-4 and $CD4^+$ T cells were detected in the resistant line compared to the non-resistant line after vaccination [55]. As IFN-γ, IL-4 and IL-17A are the effector cytokines produced by Th1, Th2 and Th17 cells, respectively [4], these results support the findings obtained in mouse models that Th1 and Th17 responses are responsible for protection against *Mycoplasma* disease. Next to that, Marchioro et al. [53] found a lower $CD4^+/CD8^+$ ratio, and thus a higher relative number of $CD8^+$ cells in pigs vaccinated with a commercial *M. hyopneumoniae* bacterin compared to control pigs receiving a physiological saline solution. This supports the hypothesis that $CD8^+$ T cells have a protective role in *Mycoplasma* infections and could partially explain the beneficial effects observed after vaccination against *M. hyopneumoniae*.

T helper 1 responses may contribute to protection against *Mycoplasma* infections by IFN-γ-mediated activation of macrophage killing. It is now well-established that Th17 immune responses are important to protect mucosal surfaces, to promote epithelial cell regeneration, mucous and antimicrobial protein production, and the release of neutrophil recruitment [56]. Following a mycoplasma infection, Th17 cells could protect the lung mucosa by attracting other immune cells for pathogen clearance [57] and by elevating secretory IgA levels in the airway lumen [58]. The major characteristic of $CD8^+$ cells is killing infected cells [59]. Since there is some evidence that *M. hyopneumoniae* is able to invade porcine epithelial cells [32], this characteristic of $CD8^+$ cells might be relevant in the immune response against *M. hyopneumoniae*. Interestingly, studies performed in the *M. pulmonis* mouse model suggest that $CD8^+$ T cells might dampen the pro-inflammatory Th cell responses responsible for lung damage and clinical disease [38]. It is suggested that IFN-γ-producing $CD8^+$ T cells may skew the responses towards a protective Th1 response. Another possibility could be that $CD8^+$ T cells kill antigen presenting cells (APCs), thus reducing the possibility of Th cell activation [38].

3 Commercial vaccines against *M. hyopneumoniae*

3.1 Type of vaccines

Commercial vaccines mostly consist of adjuvanted inactivated, whole-cell preparations (for an overview see Maes et al. [60]). Most vaccines are based on the strain J, possibly because it is the type strain of *M. hyopneumoniae* and grows easier in culture medium than recent field isolates. This strain was isolated in 1963 from a field outbreak of enzootic pneumonia in the UK. Commercial bacterin vaccines are licensed either for single or double vaccination, and combinations with porcine circovirus type 2 (PCV-2) or *Glaesserella parasuis* (formerly *Haemophilus parasuis*) are available. Most bacterin vaccines should be administered intramuscularly, but some bacterins are also licensed for intradermal administration. An inactivated vaccine based on soluble antigens of *M. hyopneumoniae* is also commercially available [61].

Attenuated vaccines against *M. hyopneumoniae* have been licensed in Mexico and in China [62]. The vaccine

in Mexico is a thermosensitive mutant of *M. hyopneumoniae* (strain LKR) that should be applied intranasally [60]. The attenuated Chinese vaccine strain is derived from a virulent parent strain 168 isolated in 1974 from an Erhua-nian pig with enzootic pneumonia [63]. This field strain was gradually attenuated by continuous alternating passage through modified Friis medium and pigs. The attenuated strain contains 60 insertions and 43 deletions compared to the original wild type strain. Mutations in genes related to metabolism and growth may contribute to the attenuated virulence, in addition to variations previously described in *M. hyopneumoniae* adhesins (P97, P102, P146, P159, P216, and LppT), cell envelope proteins (P95), cell surface antigens (P36), and secreted proteins and chaperone protein (DnaK) [36]. The Chinese vaccine strain is mostly used by intrapulmonary administration [63]. Residual virulence and/or reversion to increased virulence might represent a risk in case of attenuated vaccines, although the Chinese vaccine has been used already for many years without reported side effects [60].

3.2 Mechanisms of protection upon vaccination

Commercial vaccines induce partial protection against *M. hyopneumoniae* infections. However, the immune mechanisms resulting in partial protection are not fully elucidated. Several studies observed lower levels of the pro-inflammatory cytokines associated with lymphoid hyperplasia and pneumonia lesions, such as TNF-α, Il-6 and IL-1β in *M. hyopneumoniae*-vaccinated pigs compared to non-vaccinated ones [53, 64]. Moreover, vaccinated pigs had a higher number of IL-10-producing cells in their bronchial lymph nodes, which may have an anti-inflammatory effect [53]. Indeed, Vranckx et al. [65] demonstrated that vaccination reduces macrophage infiltration in the BALT of experimentally infected pigs. These findings suggest that vaccination modulates the infiltration of immune cells, as well as the secretion of pro- and anti-inflammatory cytokines, resulting in a reduction of lung lesions. Alternatively, it might also be possible that the reduced inflammatory responses is a consequence of a lower bacterial load [52].

The number of animals seroconverting after vaccination, as well as the antibody levels induced in serum and respiratory tract washings may vary depending on the vaccine composition, administration route, vaccination strategy (single or double vaccination) and the infection status of the animal [60]. The serological response upon a single vaccination is generally lower than after double vaccination. Serum antibodies are usually detected from two to 4 weeks after two-dose vaccination and they remain detectable for weeks to months. In the absence of natural infections that boost the immune system, antibody titers decrease below detection limits 1 to 3 months after vaccination [66]. Early studies indicate no correlation between vaccine-induced serum antibody levels and protection [67], but understanding the role of antibodies requires future investigations. Live-attenuated vaccines applied via the mucosal route could theoretically induce a local IgA response that could prevent colonization but to our knowledge, such data has not been reported.

Several studies found an increase in *M. hyopneumoniae*-specific IFN-γ-secreting cells in the blood and lung tissue of vaccinated animals [53, 64, 68]. These cells, characteristic for local and systemic Th1 responses, are considered to play an important role in vaccine-induced protection.

3.3 Efficacy of commercial vaccines

The major advantages of piglet vaccination relate to increased animal welfare and a decrease of the performance losses due to *M. hyopneumoniae* infections: improvement of daily weight gain (2–8%), feed conversion ratio (2–5%) and sometimes mortality rate. Additionally, shorter time to reach slaughter weight, less variation in slaughter weight (more homogeneous carcasses), reduced clinical signs (coughing), lower prevalence and severity of lesions typically caused by *M. hyopneumoniae* and lower treatment costs, are observed [69]. The currently used vaccines reduce the number of *M. hyopneumoniae* organisms in the respiratory tract [52, 65, 70] and decrease the infection level in a herd [71].

Different factors that may influence vaccine efficacy have been described by Maes et al. [60]. The most important factors include non-compliance with the basic principles of good vaccination practices, stress at vaccination, infections with other pathogens at the moment of vaccination, important co-infections involved in porcine respiratory disease complex, diversity of *M. hyopneumoniae* strains, and maternal immunity. A recent study [72] also hinted at a role of pre-vaccination gut microbiota composition in influencing *M. hyopneumoniae* vaccine responses, although bacterial diversity indexes alone were not predictive for antibody responses among individual pigs.

Drawbacks of the current vaccines are that the protection against clinical signs and lesions typically caused by *M. hyopneumoniae* is often incomplete and vaccination does not prevent colonization. Transmission models under experimental [70] and field conditions [45, 73] also showed that vaccination conferred only a limited and non-significant reduction of the transmission rate of *M. hyopneumoniae*. New vaccines and/or administration routes are therefore needed. A recent pilot study could not demonstrate transmission

of *M. hyopneumoniae* between seeder and contact animals in case both had been vaccinated multiple times against *M. hyopneumoniae* [74], suggesting that also the effect of vaccination frequency should be further explored.

4 Experimental vaccines

Research on the development of novel vaccines that may offer better protection against *M. hyopneumoniae* infections is ongoing. An overview of the peer-reviewed studies (since 1995) that have investigated different experimental vaccines in mice is shown in Table 1 (vector vaccines), Table 2 (subunit vaccines) and Table 3 (DNA vaccines), while studies that were conducted in pigs are shown in Table 4 (vector vaccines), Table 5 (subunit, membrane proteins, culture supernate vaccines) and Table 6 (bacterin vaccines). Peer-reviewed studies about nucleic acid-based vaccines against *M. hyopneumoniae* in pigs were not found. No publications in peer-reviewed scientific journals have been found about experimental vaccines based on attenuated strains.

4.1 Experimental vaccines studied in mice

The studies in mice focused on the construction and development of the vaccines and the evaluation of the immune responses. As *M. hyopneumoniae* is only causing disease in pigs, the efficacy of the vaccines was not tested in mice. All studies assessed the humoral responses in serum, many also included cell-mediated immunity (CMI), whereas the humoral response in the BAL fluid was less frequently examined.

In terms of antigen selection, most studies included outer membrane proteins that are immunogenic and/or considered important for adhesion such as P97, P46, P71 and P95. However, also other antigens were tested such as NrdF, P36, HSP70, P42, P37 and MnuA. NrdF is an essential enzyme for metabolic processes. It catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, an essential step in DNA replication [75]. P36 or L-lactate dehydrogenase is an early immunogenic protein [79]. HSP70 is a 70 kDA heat shock protein that has been used successfully as a vaccine antigen for other pathogens such as *Salmonella* Typhi in mice and *Mycobacterium avium* subsp. *paratuberculosis* in cattle [85]. P42 is also a heat shock protein and is a

Table 1 Experimental vector vaccines against *Mycoplasma hyopneumoniae* tested in mice.

Antigen	Vector	Route	Nb of vaccinations	Humoral response		CMI response[a]	Other/comments	References
				Serum	BALF[b]			
NrdF (R2)	*Salmonella* Typhimurium aroA SL3261	Oral	3	No IgG, no IgA	IgA, no IgG			[75]
P97 (R1)	*Salmonella* Typhimurium aroA CS332 (pro- and eukaryotic plasmid)	Oral	2	No	No	IFN-γ		[76]
NrdF (R2)	*Salmonella* Typhimurium aroA CS332(pro- and eukaryotic plasmid)	oral	2	No	No	IFN-γ response (only with eukaryotic vector)		[77]
P97 (R1)	Adenovirus	IM or IN	2	IM / IN: IgG IM: IgG2a/IgG1 = 4 IN: IgG2a/IgG1 = 1	IM /IN: IgG, IgG1, IgG2a IN: IgA		Serum and BAL inhibited growth of *M. hyopneumoniae*	[78]
P36	*Actinobacillus pleuropneumoniae* SLW36	IM	2	IgG				[79]
P97c	Adenovirus	IM	2	IgG, IgG1, IgG2a, IgG2b, IgG3 IgG2a/IgG1 ≈ 1			P97c may act as immunopotentiator	[80]
P97R1, P46	*Bacillus subtilis*	IN	2	IgG	IgA	IFN-γ, Il-4		[81]

IM, Intramuscular; IN, Intranasal.

[a] CMI cell-mediated immune responses were tested by stimulation of splenocytes.

[b] BALF bronchoalveolar lavage fluid.

Table 2 Experimental subunit vaccines against *Mycoplasma hyopneumoniae* tested in mice.

Antigen	Vaccine type	Adjuvant	Route	Nb of vaccinations	Humoral response		CMI response[a]	Other/comments	References
					Serum	BALF[b]			
P71	Subunit	*Mycobacterium tuberculosis* ESAT-6 recombinant protein	IM	2	IgG1, IgG2a IgG2a/IgG1 ≈1 (with) < 1 (without adjuvant)		IFN-γ, low Il-10		[82]
P97 (R1)	Subunit and its chimeric from with LTB[b]		IM or IN	3	IgG, IgG1, IgG2a (only with chimeric form) IM: IgG2a/IgG1 = 1.2 IN: IgG2a/IgG1 = 0.6	IgA (only with chimeric form)	IFN-γ response (only with chimeric form and IN application)	No anti-R1 antibodies with commercial bacterin	[83]
P97 (R1,R1R2)	Subunit and their chimeric forms with LTB	Montanide IMS 1113	IM	3	IgG	IgA, IgG	IFN-γ	Highest humoral response with chimeric forms	[84]
HSP70	Subunit	Nanoparticles SBa-15 and SBa-16, Aluminium	IP	3	IgG		Only in SBa-15 group; IFN-γ, Il-4, Il-10	Effect SBa-15 comparable to Aluminium	[85]
P97R1, P46, P95, P42	Chimeric – recombinant *E. coli* bacterin	Oil adjuvant (AddaVax™) (for chimeric)	IM	2	IgG, IgG1, IgG2a (IgG2a /IgG1≈0.8)			Antibodies against each antigen; higher response in chimeric group	[86]

IM, Intramuscular; IN, Intranasal; IP, Intraperitoneal.

[a] CMI responses were tested by stimulation of splenocytes.

[b] BALF bronchoalveolar lavage fluid.

[c] LTB B subunit of heat-labile enterotoxin of *E. coli*.

Table 3 Experimental DNA vaccines against *Mycoplasma hyopneumoniae* tested in mice.

Antigen	Vaccine type	Vector/ adjuvant	Route	Nb of vaccinations	Humoral response	CMI response[a]	Other/ comments	References
					Serum			
P42	DNA	pcDNA3	IM	2	IgG, IgG1, IgG2a, IgG2b, IgG3 IgG2a/ IgG1 = 1.1)	IFN-γ, Il-2, Il-4	Serum inhibited growth of *M. hyopneumoniae*	[87]
P71	DNA	*Mycobacterium tuberculosis* ESAT-6 gene sequences	IM	2	IgG1, IgG2a (higher responses and IgG2a/IgG1 ratio in group with adjuvant)	IFN-γ (higher in group with adjuvant), no Il-10		[88]
P36, P46, NrdF, and P97or P97R1	Subunit (cocktail), DNA, combination	Subunit: Aluminium pcDNA3	Subunit: SC DNA: IM	1	Subunit, combination: IgG against each antigen DNA: IgG only against P46	IFN-γ	Commercial vaccine: no anti-P97 antibodies	[89]
P37, P42, P46, P95	Subunit (cocktail) and DNA	Subunit: Aluminium pcDNA3	IM	2		IFN-γ, lower TNFα and Il-1	Strongest response for P42 and P95 (subunit) and for P46 (DNA)	[90]
P46, HSP70, MnuA antigens	Subunit (cocktail) and DNA	Subunit: Freund's adjuvant pcDNA3.1	Subunits: IP DNA: IM	3	IgG	IFN-γ, Il-10, no Il-4	Mixed response, but predominantly Th1	[91]

IM, Intramuscular; SC, Subcutaneous; IP, intraperitoneal.

[a] CMI responses were tested by stimulation of splenocytes.

member of the HSP70 family [86]. It is highly expressed under stress conditions, and specific antibodies against P42 were able to block the growth of *M. hyopneumoniae*. P37 is a lipoprotein that belongs to the ATP-binding cassette (ABC) transporters [90], whereas MnuA is a membrane nuclease and considered a potential virulence factor [91]. The antigens in the vaccines were produced by recombinant DNA technology using *E. coli* as expression system.

Most vector vaccines were based on bacterial vectors namely *Salmonella*, *Actinobacillus* and *Bacillus*, while two studies used adenovirus vectors. The subunit vaccines were based on single antigens or different antigens that were mixed with an adjuvant. In three of the five studies, the antigens were constructed as a chimeric protein. Subunit vaccines were used in combination with adjuvants based on oil, aluminium hydroxide, the B subunit of heat-labile enterotoxin of *E. coli* (LTB) or *Mycobacterium tuberculosis* ESAT-6 protein. Vaccination with DNA constructs encoding potential antigens might be promising, as in general, DNA constructs are stable, easy to handle and can be administered via various routes.

Most vaccines in mice were applied two or three times, and were administered via the parenteral route, mainly intramuscularly, although oral (vector vaccines), intranasal and intraperitoneal administration routes were evaluated as well.

The major outcome parameters of the studies in mice were humoral responses in serum and in some studies also in BAL fluid, and CMI responses. Humoral responses in serum were assessed by measuring IgG against the antigens included in the vaccine, some studies also measured isotypes of IgG. In general, IgG1 in mice is indicative of a Th2 response, whereas IgG2a is predominantly produced during a Th1-type response [92]. CMI responses were examined by isolation and subsequent stimulation of splenocytes with the respective antigens. IFN-γ production by splenocytes was measured in every study, other cytokines (IL-10, IL-4, TNF-α) were less frequently assessed.

The immune responses with the orally applied *Salmonella* vector vaccine were poor, IgA was only found in BAL fluid in one study and IFN-γ production in another study (Table 1). The immune responses with the other vector vaccines that were administered intramuscularly or intranasally were more pronounced, but also variable between studies. When used under field conditions, vector vaccines might have the problem that immunity is present in the animals against

Table 4 Efficacy and immune responses of experimental vector vaccines against *Mycoplasma hyopneumoniae* tested in pigs.

Antigen	Vector	Route	Number of vaccinations	Significant decrease of			Humoral response		CMI response[a]	Other/comments	References
				Lung lesions	Clinical signs	*M. hyopneumoniae* numbers	Serum	BALF[b]			
NrdF (R2)	*S.* Typhimurium aroA SL3261[c]	Oral	2	Yes			No	IgA	Yes	Higher ADG[b]; IgA only after challenge	[94]
P97 (R1R2)	*E. rhusiopathiae* strain YS-19[c]	Intranasal	2	Yes		Yes	No		Yes		[95]
P97 (R1R2)	*E. rhusiopathiae* strain Koganei 65–0.15	Oral	3	Yes		No	IgG	IgA	Yes	IgG and IgA only after challenge	[96]
P97 (R1R2)	Adenovirus	Intranasal	2	Yes		Yes	IgG, IgA	IgA, IgG (saliva)	Yes	higher ADG; serum inhibited *M. hyopneumoniae* growth; minor decrease of lung inflammation Commercial vaccine: no anti-P97 antibodies, better protection	[97]

[a] CMI responses were tested by stimulation of peripheral blood mononuclear cells (PBMCs) and calculating stimulation indexes.

[b] BALF, bronchoalveolar lavage fluid; ADG, average daily gain.

[c] *S.* Typhimurium: *Salmonella* Typhimurium; *E. rhusiopathiae: Erysipelothrix rhusiopathiae.*

Table 5 Efficacy and/or immune responses of experimental vaccines (subunit, membrane proteins, culture supernate) against *Mycoplasma hyopneumoniae* tested in pigs.

Antigen	Vaccine type	Adjuvant/ carrier	Route	Nb of vaccinations	Challenge infection	Decrease of			Humoral response		CMI response[b]	Other/ comments	References
						Lung lesions	Clinical signs	*M. hyopneumoniae* numbers	Serum	BALF[c]			
P97	Subunit	Complete Freund's adjuvant	IM	2	Yes	No	No		Yes[d]				[98]
J strain	Membrane proteins	Five different adjuvants[e]	IM-IP	2	Yes	Yes[e]			IgG, IgA	IgA, IgG		No effect on ADG; Humoral response in BALF: only after challenge, greater in IM groups	[48]
Strain 1986–1	Cell-free culture supernate	Al(OH)3	IM	2	Yes	Yes		Yes	IgG (low)				[99]
Strain 1986–1	Cell-free culture supernate	Al(OH)3	IM	2	Yes	Yes		Yes		IgA, IgG		Less macrophages, lymphocytes and TNFα in lungs Humoral response in BALF only after challenge	[100]
P97 (RR1)[a]	Chimeric subunit with *Pseudomonas* exotoxin A		SC (mice)-IM (pigs)	2	No				IgG				[101]
P97 (R1R2)	Chimeric subunit with N-terminal region *A. pleuropneumoniae* ApxIII	Freund's adjuvant (mice), Al(OH)3 (pigs)	SC (mice)-IM (pigs)	2	Yes (pigs)	Yes	Yes		IgG1, IgG2a (mice)		Higher IFN-γ and IL-4 (mice)	Also protection against *A. pleuropneumoniae*	[102]

Table 5 (continued)

Antigen	Vaccine type	Adjuvant/ carrier	Route	Nb of vaccinations	Challenge infection	Decrease of			Humoral response		CMI response[b]	Other/ comments	References
						Lung lesions	Clinical signs	*M. hyopneumoniae* numbers	Serum	BALF [c]			
P97, P42, NrdF	Chimeric subunit	LTB[f]	IM-IN	2	Yes	No	No	No	IgG	IgA		IgA in BALF only after challenge Commercial vaccine: lower serological response, better protection	[21]
P102 and 8 fragments of P97 / P102 paralogs	Subunit	Al(OH)3 and polymer based (Montanide™)	IM	3	Yes	No	No	No	IgG	No IgA		Less cilia damage, less Il-1, Il-6, TNFα in BALF Commercial vaccine: lower serological response, better protection	[52]

IM, Intramuscular; SC, Subcutaneous; IN, Intranasal; IP, intraperitoneal.

[a] Study was done in pigs and mice.

[b] CMI response was tested by stimulation of splenocytes.

[c] BALF bronchoalveolar lavage fluid.

[d] tested by Western blotting for reactivity with whole cell lysates.

[e] The membrane preparations were formulated with one of the following adjuvants: 1) Auspharm oil, 2) Alhydrogel, 3) Algammulin, 4) DEAE dextran with Auspharm oil, 5) DEAE dextran with mineral oil. Lung lesions were significantly reduced with all formulations compared to non-vaccinated pigs, but there were no significant differences between the formulations.

[f] LTB B subunit of heat-labile enterotoxin of *E. coli*.

Table 6 Efficacy and/or immune responses of experimental bacterin vaccines against *Mycoplasma hyopneumoniae* tested in pigs.

Mycoplasma strain	Adjuvant/ carrier	Route	Nb of vaccinations	Challenge infection	Decrease of			Humoral response		CMI response[a]	Other/ comments	References
					Lung lesions	Clinical signs	*M. hyopneumoniae* numbers	Serum	BALF[b]			
PRIT-5	Micro-encapsulated (oral); Al(OH)3 (IM)	Oral – IM	3	Yes	Yes			IgG, IgA	IgA (nose, saliva)		Highest humoral responses in oral group	[103]
PRIT-5	Micro-encapsulated (oral); Al(OH)3 (IM)	Oral – IM – IM/ oral	3	Yes	Yes			IgG, IgA	IgA (nose, saliva)		Highest humoral responses in oral/ combined groups Best protection in IM/ oral group	[104]
F7.2C [c]	Lipo_AMP	IM-IM	2	No				IgG, no IgA	no IgA	Th1 – no Th17	E: 3/3 – L: 3/3 [d]	[105]
	Lipo_TLR	IM-IM								no Th1 – no Th17	E: 3/3 – L: 3/3	
	Lipo_DDA:TDB	ID-IM								Th1 – no Th17	E: 2/3 – L: 1/3	
	SWE_TLR	IM-IM								Th1 – no Th17	E: 3/3 – L: 1/3	
	PLGA_TLR	IM-IM						No IgG/IgA	no IgA	no Th1 – Th17	E: 0/3 – L: 0/3	
F7.2C	Lipo_DDA:TDB	IM-IM	2	Yes	Yes	Yes	Yes	IgG, IgA	IgA	Th1, Th17, CD8+	Also reduction of microscopic lung lesions IgA response only after challenge Highest efficacy in SWE_TLR group	[106]
	SWE_TLR									Th1, Th17, CD8+		
	PLGA_TLR									Th1, no Th17, CD8+		

IM, Intramuscularly; ID, intradermally.

[a] CMI responses were tested by stimulation of peripheral blood mononuclear cells (PBMCs).

[b] BALF bronchoalveolar lavage fluid.

[c] The bacterin was formulated with 1) cationic liposomes + STING ligand c-di-AMP (Lipo_AMP), 2) cationic liposomes with TLR ligands targeting TLR1/2, TLR7/8 and TLR9 (Lipo_TLR), 3) cationic liposome formulation with the MINCLE agonist trehalose 6,6-dibehenate DDA:TDB liposomes (Lipo_DDA:TDB), 4) squalene-in-water emulsion with the same TLR ligands (SWE_TLR), 5) microparticle formulation with the same TLR ligands (PLGA_TLR).

[d] Number of induced blood transcriptional modules (BTM) by the vaccine group. In total, three early (E) (day 0 to 1) and three late (L) (day 1 to 7) different BTM) were measured: early inflammatory, early IFN type I, early myeloid cell/DC, late cell cycle, late T/NK-cell, late Ig.

the vector micro-organism e.g. *Salmonella*, leading to lower expression of and immunity against the carried antigen.

The immune responses following administration of subunit vaccines (Table 2) were more consistently measured compared to those of the vector vaccines. Mostly, mixed Th1/Th2 responses were obtained. Immune responses were generally more pronounced when different antigens were presented in a chimeric form [83, 84, 86]. Possibly, in these studies, the epitopes were better accessible by the immune system in the chimeric proteins. Immunization by the intramuscular route appeared to favor a Th1-type response (higher IgG2/IgG1 ratio), while the intranasal route induced a mixed response [78, 83]. Adding *Mycobacterium tuberculosis* ESAT-6 protein or the gene sequences as adjuvant also shifted the immune response towards a Th1-type response [82].

Vaccination with the DNA constructs elicited mixed responses (Table 3), with predominance for Th1-type responses [87, 91]. IgA in BAL fluid was not tested. Th1-type responses stimulate B-cells to produce strongly opsonizing antibodies, such as IgG2a and IgG2b in mice, enhancing the phagocytosis by alveolar macrophages. In mice, IFN-γ, produced by Th1-type cells, induces nitric oxide release from monocytes/macrophages. In case genes coding for multiple antigens were included [89–91], the responses were variable between the antigens, and could also be different when compared to the response obtained by the respective single subunit vaccines.

Overall, some studies showed promising immune responses in mice. However, many phenotypic as well as functional differences in immune cell populations exist between the porcine and the murine immune system [93]. Therefore, studies in mice should be considered as preliminary and need to be validated in pigs.

4.2 Experimental vaccines studied in pigs

The experimental vaccine studies in pigs are summarized in Tables 4, 5 and 6. Most of the studies in pigs used an experimental challenge infection model, allowing to assess the efficacy of the vaccines. CMI responses in pigs were tested by stimulation of PBMCs and subsequently measuring the stimulation index (vector vaccines) or specific cytokine responses (bacterin vaccines). No CMI responses were measured in the studies with the subunit vaccines.

The antigen selection for the vector and subunit vaccines mostly included the P97, alone or in combination with other antigens e.g. NrdF, P42 or P102. One study used membrane proteins [48] and two studies used cell-free culture supernatant of *M. hyopneumoniae* [99, 100]. The bacterin vaccines were based on the strains PRIT-5 [103, 104] and F7.2C [105, 106]. Subunit and bacterin vaccines were mostly used in combination with an adjuvant such as aluminium hydroxide, Freund's adjuvant (both complete or incomplete), bacterial toxin subunits (LTB), oil or polymer-based adjuvants, macrophage-inducible C-type lectin (MINCLE) agonist, the stimulator of interferon genes (STING) ligand and a combination of different TLR ligands. Vectors used were *Salmonella* Typhimurium, *Erysipelothrix rhusiopathiae* and adenovirus. Vector vaccines were administered orally or intranasally, while most subunit and bacterin vaccines were administered parenterally. In two studies, the bacterin was provided orally in microspheres [103, 104]. All experimental vaccines in pigs were applied two or three times.

In terms of efficacy, 11 out of 14 studies showed a significant reduction of the lung lesions caused by *M. hyopneumoniae* upon challenge infection. Two out of five studies showed a reduction of clinical signs (coughing), and five out of eight a reduction of the *M. hyopneumoniae* load in the BAL fluid. Three studies [21, 52, 97] included a commercial bacterin as control. Their efficacy was each time better than for the experimental vaccines, although the immune responses against the epitopes of the experimental vaccines were lower or absent upon vaccination with the commercial vaccines. This might be due to the fact that some proteins of the bacterins might not be expressed or only expressed in negligible amounts under culture conditions [107], or to the formulation or the adjuvant. Proteomic analysis by Pendarvis et al. [108] showed that only 483 of 691 (70%) proteins in *M. hyopneumoniae* 232 strain were expressed under in vitro culture conditions. This might be one of the reasons of the incomplete protection induced by commercial vaccines. Nonetheless, the efficacy of the experimental vaccines was even lower, suggesting that immunity induced by the antigens selected for preparing these vaccines is not sufficient for improved protection, although it is well known that these antigens like P97 have important roles in the pathogenesis of *M. hyopneumoniae* infections.

All studies could demonstrate specific IgG responses in the serum, except for two vector vaccines [94, 95]. In all studies that had measured mucosal IgA responses in the BAL fluid, IgA could only be detected after challenge infection, but not after vaccination prior to challenge (four studies used intramuscular, two studies oral administration). In three studies [97, 103, 104], IgA was detected in the saliva and/or nose after vaccination and prior to challenge infection. In these studies, pigs were vaccinated intranasally, orally or intramuscularly. In the pigs vaccinated intramuscularly, IgA levels in the saliva and especially in the nose were low [103, 104].

In terms of CMI responses, all studies with the vector vaccines could demonstrate significant responses upon specific stimulation of PBMCs. None of the studies with subunit vaccines (Table 5) describe assessment of CMI responses. For the bacterin vaccines, CMI responses were only assessed in the studies of Matthijs et al. [105, 106]. The study of Matthijs et al. [105] did not apply challenge infection, but made a detailed assessment of the immune responses of five different bacterin formulations. They were all based on a more recently isolated field strain of *M. hyopneumoniae* compared to the type strain J, in combination with different novel adjuvants. These included different Toll-like receptor ligands (targeting TLR1/2, TLR7/8, TLR9), the MINCLE agonist trehalose 6,6-dibehenate (TDB), and STING ligand cyclic diadenylate monophosphate (c-di-AMP). They were used in combination with different carriers namely liposomes, polylactic-co-glycolic acid (PLGA) microparticles or squalene-in-water emulsion (SWE) (see Table 6). The responses were variable depending on the group. Lipo_DDA:TDB, Lipo_AMP and SWE_TLR significantly induced Th1 cytokine-secreting T cells. Only PLGA_TLR appeared to induce Th17 cells, but was unable to induce serum antibodies.

The study of Matthijs et al. [106] also applied a systems vaccinology approach developed for humans and adapted the approach for use in pigs. The transcriptomic analyses demonstrated that the induction of inflammatory and myeloid cell blood transcriptional modules (BTM) in the first 24 h after vaccination correlated well with serum antibodies, while negative correlations with the same modules were found seven days post vaccination. Furthermore, many cell cycle and T cell BTM upregulated at day seven, correlated positively with adaptive immune responses. When comparing the delivery of the identical TLR ligands with the three formulations, SWE_TLR was shown to be more potent in the induction of an early innate immune response, while the liposomal formulation more strongly promoted late cell cycle and T cell BTM. For the PLGA formulation, there were signs of a delayed and weak perturbation of these BTM. The study of Matthijs et al. [105] demonstrated the utility of transcriptome-based systems immunology analyses in identifying early immune signatures in the blood and in unraveling the mechanistic events leading to the stimulation of adaptive immune responses after vaccine injection in pigs.

In a subsequent study, Matthijs et al. [106] assessed the efficacy of three bacterin formulations that were able to induce a Th1 or Th17 response in the previous study, namely a cationic liposome formulation with the Mincle receptor ligand trehalose 6,6-dibehenate (Lipo_DDA:TDB), a squalene-in-water emulsion with Toll-like receptor (TLR) ligands targeting TLR1/2, TLR7/8 and TLR9 (SWE_TLR), and a poly(lactic-co-glycolic acid) microparticle formulation with the same TLR ligands (PLGA_TLR). All three formulations showed promising results, but the highest CMI responses were obtained in the SWE_TLR group. This experimental vaccine also showed the best efficacy in terms of reducing clinical signs, lung lesions and bacterial load in the lung.

Overall, the research with the different experimental vaccines in pigs suggests that the construction, the type of antigens, the adjuvant and/or carrier, and the route and frequency of immunization could induce variable immune responses and efficacy. Based on the available studies, it is not possible to unravel the effect of one of these characteristics e.g. antigen, adjuvant and administration route, as the studies were not designed for this purpose and differ each time in more than one characteristic. In addition, the studies listed in Tables 1 and 2 also point to a large variation in individual animal responses, even if the same vaccine is used under the same conditions.

5 Avenues for further research

As far as this was investigated, the experimental vaccines that have been tested in pigs (Tables 4, 5, 6) do not provide superior protection compared to the commercially available bacterin vaccines. Therefore, further research on the development of new experimental vaccines and validation of the efficacy of promising vaccines in pigs is needed.

From an immunological point of view, a major challenge is to induce a protective immunity at the mucosal surface of primary adherence and multiplication of *M. hyopneumoniae*, namely the ciliated epithelium of the trachea, bronchi and bronchioli. From a practical viewpoint, also aspects such as cost of the vaccine, ease of production, transport and administration, differentiation of infected from vaccinated animals (DIVA), and possible combination with other vaccines are important.

5.1 Administration route

Administration of the antigens intranasally or via aerosols might be the most appropriate routes to accomplish mucosal protection. The number of experimental vaccines that have evaluated this route of immunization is still very limited. This is mainly due to important difficulties that are encountered by using the mucosal route such as: (1) the antigen should be present at and below the mucosal barrier in sufficient amounts allowing sufficient capturing by antigen presenting cells, (2) mucosal tolerance mechanisms should be overcome, (3) protective immune mechanisms should be activated and, (4) minimal/or no influence on mucosal functionality should

occur. Mucosal adjuvants e.g. microbe-derived substances, including TLR ligands, could be crucial in reaching these goals [109, 110]. Heterologous prime-boost regimes e.g. intramuscular followed by mucosal administration or vice versa, allowing a broader or more variable triggering of the immune system, might also be considered. Vaccination via aerosols is probably technically possible, as nebulization of lung homogenate positive for *M. hyopneumoniae* to gilts under field conditions, resulted in infection of the animals with this pathogen [111]. Also, Feng et al. [62] showed that the attenuated vaccine used in China could be administrated as an aerosol.

Providing the antigen orally might also induce mucosal immunity in the respiratory tract via the common mucosal immune system [112]. In the studies of Lin et al. [103, 104], microencapsulated inactivated whole-cell *M. hyopneumoniae* vaccine was administered orally to pigs via a tube into the esophagus on three occasions. IgA was detected in nasal secretions and saliva, but IgA was not measured in BAL fluid. However, lung lesions following challenge infection were significantly reduced in vaccinated pigs, pointing to protective immunity in these animals. Placing a tube in the esophagus is labor intensive, time consuming and not animal welfare friendly. The stability of the antigen during the mouth-stomach-intestine passage might also be an issue. Therefore, further research should focus on whether *M. hyopneumoniae* vaccines mixed in the diet and consumed by the pigs can also provide protection. Such a method might require a higher antigen dose and also the stability of the antigen in the feed might be an issue. However, it would be animal welfare friendly and require much less time and labor than administration of the vaccine via a tube to each individual animal.

Most experimental vaccines were applied via the parenteral route, mostly intramuscularly. The studies using parenteral administration however could not demonstrate measurable IgA responses in the BAL fluid prior to challenge, and therefore, this route is less suitable for inducing mucosal humoral responses. However, higher *M. hyopneumoniae*-specific IgA levels were observed in respiratory tract washings of parenterally vaccinated pigs compared to non-vaccinated pigs after challenge infection, indicating an anamnestic immune response. This suggests that priming of the mucosal immune system is possible after parenteral vaccine administration. Intradermal vaccination directly targets epidermal Langerhans cells and dermal dendritic cells, which are essential for efficient T and B cell priming. In this sense, intradermal vaccination against *M. hyopneumoniae* can be an asset, as more of these specialized APCs are present in the skin compared to muscle tissue [113]. In addition, no needles are used as the vaccine is administered intradermal via pressure, which may reduce the risk for iatrogenic infections.

5.2 Antigen

Bacterial genes and antigens involved in survival of the bacterium in the host and/or that render the bacterium harmful to the host could be included in vaccines or could be targets for attenuation to develop attenuated vaccines. Many experimental vaccine studies have included P97 and other proteins that are important for adherence or metabolism. However, subunit vaccines based on the most important adhesion namely P97, did not provide sufficient protection. The adhesion process is complex and involves many different adhesins, therefore, including one or only a few antigens might be insufficient. This might also explain why bacterins, which include a broad array of antigens, perform better in terms of efficacy than subunit vaccines. Further research on subunit vaccines should therefore not focus on one or a few antigens, but include different and carefully selected antigens.

5.3 Adjuvant and carrier

Adjuvants are incorporated into vaccines to increase and direct the immunogenic responses to antigens. Many adjuvants activate the innate immune system through pattern-recognition receptors (PRRs) present in immune cells. Receptor-ligand interactions lead to expression of genes that encode cytokines, chemokines, and costimulatory molecules responsible for priming, expansion, and polarization of immune responses [114]. Adjuvants can also induce adaptive immune responses either by enhancing T cell responses, by stimulating humoral immunity or both. As Th1 and Th17 responses are considered important for protection against *M. hyopneumoniae* infection, adjuvants that stimulate this arm of the immune system should preferably be selected [105]. Commonly used adjuvants and their modes of action along with strengths and weaknesses have been reviewed by Bastola et al. [114].

Adjuvants not only increase the immunogenic responses, but can also lead to adverse reactions such as inflammation at the injection site, granuloma and sterile abscess formation, malaise, fever and other systemic reactions. Apart from the study of Matthijs et al. [105], information about safety of adjuvants in experimental *M. hyopneumoniae* vaccines is very scarce. Research on adjuvant development should therefore not only identify more inflammatory adjuvants, but also try to separate the potency of adjuvants from the adverse effects they can induce.

5.4 Attenuated vaccines

Attenuated vaccines have great potential to better stimulate a mucosal immune response at the respiratory tract compared to parenterally administered vaccines. However, apart from the two current commercially available attenuated *M. hyopneumoniae* vaccines, very few studies have focused on developing experimental attenuated *M. hyopneumoniae* vaccines. This might be due to the difficult isolation and cultivation characteristics of *M. hyopneumoniae*. In addition, reversion of virulence is always a concern in case of attenuated vaccines. One study [115] tested whether a low virulent *M. hyopneumoniae* strain could be used as a potential vaccine. However, infection with this strain did not protect piglets against infection with a highly virulent *M. hyopneumoniae* isolate 1 month later, suggesting that low virulent strains might not be suitable as such to be used as vaccines. Current technology allows however to selectively delete specific genes important for replication or virulence in pathogenic organisms, which might be useful for the development of attenuated vaccines against *M. hyopneumoniae*. At this stage however, these genes are not yet fully elucidated, and therefore, further research is needed to investigate genes of *M. hyopneumoniae* involved in pathogenesis and virulence.

6 Conclusions

The very complex interaction of *M. hyopneumoniae* with the respiratory tract and the immune system and the fact that naturally elicited immune responses upon infection are not able to rapidly clear the pathogen from the animal, make the search for protective immune responses and efficacious novel vaccines challenging. Different experimental vaccines against *M. hyopneumoniae* have been developed and tested in mice and pigs. Most of them were based on the P97 adhesin or other factors considered important in the pathogenesis, or were bacterins combined with novel adjuvants. As cell-mediated and likely also mucosal humoral responses are important for protection, new vaccines should aim to target these arms of the immune response. Very few research has been directed towards the development of attenuated vaccines, although such vaccines may have great potential.

Abbreviations

ABC: ATP-binding cassette; APC: Antigen presenting cell; BAL: Bronchoalveolar lavage; BTM: Blood transcriptional modules; c-di-AMP: Cyclic di-adenylate monophosphate; CMI: Cell-mediated immunity; DC: Dendritic cell; DDA:TDB: Dimethyl dioctadecylammonium:trehalose 6,6-dibehenate; DIVA: Differentiation of infected from vaccinated animals; HSP: Heat shock protein; ICE: Integrative and conjugative elements; IFN: Interferon; Ig: Immunoglobulin; IL: Interleukin; IN: Intranasal; LAMP: Lipid associated membrane proteins; LTB: Heat-labile enterotoxin of *E. coli*; *M. hyopneumoniae*: *Mycoplasma hyopneumoniae*; MET: Macrophage extracellular trap; MIB: *Mycoplasma* Ig binding protein; MINCLE: Macrophage-inducible C-type lectin; MIP: *Mycoplasma* Ig protease; NET: Neutrophil extracellular trap; NK: Natural killer; NrdF: Ribonucleoside-diphosphate reductase; PBMC: Peripheral blood mononuclear cell; PGLA: Poly(lactic-*co*-glycolic acid); STING: Stimulator of interferon genes; SWE: Squalene-in-water; Th: T helper; TLR: Toll-like receptor; TNF: Tumor necrosis factor.

Authors' contributions

DM wrote the paper. All co-authors have critically revised the manuscript, FB, PK and FH with emphasis on the bacteriological aspects, BV and AS with emphasis on the immune responses and vaccination. The authors have collaborated intensively on the topic during the H2020 Project SAPHIR (Project No. 633184) (2015–2019). All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium. [2]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland. [3]Institute of Virology and Immunology, Sensemattstrasse 293, Mittelhäusern, Switzerland. [4]Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland.

Received: 17 February 2021 Accepted: 23 April 2021
Published online: 08 May 2021

References

1. Pieters M, Maes D (2019) Mycoplasmosis. In: Zimmermann JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J Diseases of swine (11 ed). Hoboken, NJ, Wiley. pp: 863–883
2. Marois C, Segalés J, Holtkamp D, Chae C, Deblanc C, Opriessnig T, Fablet C (2020) Interactions of *Mycoplasma hyopneumoniae* with other pathogens and economic impact. In: Book Mycoplasmas in Swine. Chapter 7. Editors: Dominiek Maes, Marina Sibila, Maria Pieters. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium, 127–145
3. Blanchard B, Vena MM, Cavalier A, Le Lannic J, Gouranton J, Kobisch M (1992) Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. Vet Microbiol 30:329–341
4. Summerfield A (2020) Immune responses against porcine *Mycoplasma* infections. In: Book Mycoplasmas in Swine. Chapter 6. Editors: Dominiek Maes, Marina Sibila, Maria Pieters. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium, pp. 110–125
5. Marco E, Yeske P, Pieters M (2020) General control measures against *Mycoplasma hyopneumonia*e infections. In: Book Mycoplasmas in Swine. Chapter 9. Editors: Dominiek Maes, Marina Sibila, Maria Pieters. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp. 163–180
6. Thacker E, Thacker B, Wolff T (2004) Efficacy of a chlortetracycline feed additive in reducing pneumonia and clinical signs induced by experimental *Mycoplasma hyopneumoniae* challenge. J Swine Health Prod 14:140–144
7. Collignon PJ, McEwen SA (2019) One health-its importance in helping to better control antimicrobial resistance. Trop Med Infect Dis 4:22
8. Jarocki V, Djordjevic S (2020) Diversity of *Mycoplasma hyopneumoniae* strains. In: Book Mycoplasmas in Swine. Chapter 2. Editors: Maes D, Sibila M, Pieters M. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp. 47–71
9. Minion FC, Lefkowitz E, Madsen M, Cleary B, Swartzell S, Mahairas G (2004) The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. J Bacteriol 186:7123–7133
10. Vasconcelos A, Ferreira H, Bizarro C, Bonatto S, Carvalho M, Pinto P, Almeida DF, Almeida LGP, Almeida R, Alves-Filho L, Assunção EN, Azevedo VAC, Bogo MR, Brigido MM, Brocchi M, Burity HE, Camargo AA, Camargo SS, Carepo MS, Carraro DM, de Mattos Cascardo JC, Castro LA, Cavalcanti G, Chemale G, Collevatti RG, Cunha CW, Dallagiovanna B,

Dambrós BP, Dellagostin OA et al (2005) Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*. J Bacteriol 187:5568–5577
11. Leal Zimmer F, Paes J, Zaha A, Ferreira H (2020) Pathogenicity & virulence of *Mycoplasma hyopneumoniae*. Virulence 11:1600–1622
12. Felde O, Kreizinger Z, Sulyok KM, Hrivnák V, Kiss K, Jerzsele A, Biksi I, Gyuranecz M (2018) Antibiotic susceptibility testing of *Mycoplasma hyopneumoniae* field isolates from Central Europe for fifteen antibiotics by microbroth dilution method. PLoS One 13:e0209030
13. Fritsch TE, Siqueira FM, Schrank IS (2015) Intrinsic terminators in *Mycoplasma hyopneumoniae* transcription. BMC Genomics 16:273–284
14. Rycroft A (2020) Overview of the general characteristics and classification of porcine *Mycoplasma* species. In: Book Mycoplasmas in Swine. Chapter 1. Editors: Maes D, Sibila M, Pieters M. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp. 25–46
15. Simionatto S, Marchioro SB, Galli V, Luerce T, Hartwig D, Moreira A, Dellagostin O (2009) Efficient site-directed mutagenesis using an overlap extension-PCR method for expressing *Mycoplasma hyopneumoniae* genes in *Escherichia coli*. J Microbiol Methods 79:101–105
16. Debey MC, Ross RF (1994) Ciliostasis and loss of cilia induced by *Mycoplasma hyopneumoniae* in porcine tracheal organ cultures. Infect Immun 62:5312–5318
17. Maes D, Sibila M, Kuhnert P, Segalés J, Haesebrouck F, Pieters M (2018) Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. Transbound Emerg Dis 65:110–124
18. Kuhnert P, Jores J (2020) *Mycoplasma hyopneumoniae* pathogenicity: the know and the unknown. In: Book Mycoplasmas in Swine. Chapter 3. Editors: Maes D, Sibila M, Pieters M. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp. 71–84
19. Raymond BB, Jenkins C, Seymour LM, Tacchi J, Widjaja M, Jarocki V, Deutscher A, Turnbull L, Whitchurch C, Padula M, Djordjevic S (2015) Proteolytic processing of the cilium adhesion MHJ_0194 (P123J) in *Mycoplasma hyopneumoniae* generates a functionally diverse array of cleavage fragments that bind multiple host molecules. Cell Microbiol 17:425–444
20. Jenkins C, Wilton JL, Minion FC, Falconer L, Walker MJ, Djordjevic SP (2006) Two domains within the *Mycoplasma hyopneumoniae* cilium adhesin bind heparin. Infect Immun 74:481–487
21. Marchioro S, Del Pozo SR, Michiels A, Haesebrouck F, Conceição F, Dellagostin O, Maes D (2014) Immune responses of a chimeric protein vaccine containing *Mycoplasma hyopneumoniae* antigens and LTB against experimental *M. hyopneumoniae* infection in pigs. Vaccine 32:4689–4694
22. Kucharewicz I, Kowal K, Buczko W, Bodzenta-Lukaszyk A (2003) The plasmin systemin airway remodelling. Thromb Res 112:1–7
23. Raymond B, Madhkoor R, Schleicher I, Uphoff C, Turnbull L, Whitchurch C, Rohde M, Padula M, Djordjevic S (2018) Extracellular actin is a receptor for *Mycoplasma hyopneumoniae*. Front Cell Infect Microbiol 8:54
24. Paes JA, Machado LDPN, dos Anjos Leal FM, de Moraes SN, Moura H, Barr JR, Ferreira HB (2018) Comparative proteomics of two *Mycoplasma hyopneumoniae* strains and *Mycoplasma flocculare* identified potential porcine enzootic pneumonia determinants. Virulence 9:1230–1246
25. Ferrarini MG, Mucha SG, Parrot D, Meiffrein G, Ruggiero Bachega JF, Comte G, Zaha A, Sagot MF (2018) Hydrogen peroxide production and myo-inositol metabolism as important traits for virulence of *Mycoplasma hyopneumoniae*. Mol Microbiol 108:683–696
26. Zuo LL, Wu YM, You XX (2009) *Mycoplasma* lipoproteins and Toll-like receptors. J Zhejiang Univ Sci B 10:67–76
27. Bai F, Ni B, Liu M, Feng Z, Xiong Q, Shao G (2015) *Mycoplasma hyopneumoniae*-derived lipid-associated membrane proteins induce inflammation and apoptosis in porcine peripheral blood mononuclear cells *in vitro*. Vet Microbiol 175:58–67
28. Liu M, Du G, Liu B, Hu Y, Liu J, Jia Y, Minion FC, Shao G, Zhao R (2017) Cholesterol exacerbates *Mycoplasma hyopneumoniae*-induced apoptosis via stimulating proliferation and adhesion to porcine alveolar macrophages. Vet Microbiol 211:112–118
29. Mitiku F, Hartley CA, Sansom FM, Coombe JE, Mansell PD, Beggs DS, Browning GF (2018) The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*. Vet Microbiol 218:13–19
30. Henthorn CR, Minion CF, Sahin O (2018) Utilization of macrophage extracellular trap nucleotides by *Mycoplasma hyopneumoniae*. Microbiology 164:1394–1404
31. Li P, Zhang Y, Li X, Zhou W, Li X, Jiang F, Wu W (2019) *Mycoplasma hyopneumoniae* Mhp597 is a cytotoxicity, inflammation and immunosuppression associated nuclease. Vet Microbiol 235:53–62
32. Raymond B, Turnbull L, Jenkins C, Madhkoor R, Schleicher I, Uphoff C, Whitchurch C, Rohde M, Djordjevic S (2018) *Mycoplasma hyopneumoniae* resides intracellularly within porcine epithelial cells. Sci Rep 8:17697
33. Grover RK, Zhu X, Nieusma T, Jones T, Boreo I, MacLeod A, Mark A, Niessen S, Kom H, Kong L, Assad-Garcia N, Kwon K, Chesi M, Smider V, Salomon D, Jelinek D, Kyle R, Pyles R, Glass J, Ward A, Wilson I, Lerner R (2014) A structurally distinct human mycoplasma protein that generically blocks antigen-antibody union. Science 343:656–661
34. Arfi Y, Minder L, Di Primo C, Le-Roy A, Ebel C, Coquet L, Claverol S, Vashee S, Jores J, Blanchard A, Sirand-Pugnet P (2016) MIB-MIP is a mycoplasma system that captures and cleaves immunoglobulin G. Proc Natl Acad Sci U S A 113:5406–5011
35. Pinto PM, de Carvalho MO, Alves-Junior L, Brocchi M, Schrank IS (2007) Molecular analysis of an integrative conjugative element, ICEH, present in the chromosome of different strains of *Mycoplasma hyopneumoniae*. Genet Mol Biol 30:256–263
36. Liu W, Xiao S, Li M, Guo S, Li S, Luo R, Feng Z, Li B, Zhou Z, Shao G, Chen H, Fang L (2013) Comparative genomic analyses of *Mycoplasma hyopneumoniae* pathogenic 168 Strain and its high-passaged attenuated strain. BMC Genomics 14:80
37. Browning GF, Marenda MS, Noormohammadi AH, Markham PF (2011) The central role of lipoproteins in the pathogenesis of mycoplasmoses. Vet Microbiol 153:44–50
38. Dobbs NA, Odeh AN, Sun X, Simecka JW (2009) The multifaceted role of T cell-mediated immunity in pathogenesis and resistance to *Mycoplasma* respiratory disease. Curr Trends Immunol 10:1–19
39. Lorenzo H, Quesada O, Assuncao P, Castro A, Rodriguez F (2006) Cytokine expression in porcine lungs experimentally infected with *Mycoplasma hyopneumoniae*. Vet Immunol Immunopathol 109:199–207
40. Muneta Y, Uenishi H, Kikuma R, Yoshihara K, Shimoji Y, Yamamoto R, Hamashima N, Yokomizo Y, Mori Y (2003) Porcine TLR2 and TLR6: identification and their involvement in *Mycoplasma hyopneumoniae* infection. J Interferon Cytokine Res 23:583–590
41. Okusawa T, Fujita M, Nakamura J, Into T, Yasuda M, Yoshimura A, Shibata K (2004) Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. Infect Immun 72:1657–1665
42. Trueeb B, Braun R, Auray G, Kuhnert P, Summerfield A (2020) Differential innate immune responses induced by *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* in various types of antigen presenting cells. Vet Microbiol 240:108541
43. Mucha S, Ferrarini M, Moraga C, De Genova A, Guyon L, Tardy F, Rome S, Sagot MF, Zaha A (2020) *Mycoplasma hyopneumoniae* J elicits an antioxidant response and decreases the expression of ciliary genes in infected swine epithelial cells. Sci Rep 10:13707
44. Kobisch M, Blanchard B, Le Potier MF (1993) *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to reinfection. Vet Res 24:67–77
45. Villarreal I, Meyns T, Haesebrouck F, Dewulf J, Vranckx K, Calus D, Pasmans F, Maes D (2011) Effect of vaccination against *Mycoplasma hyopneumoniae* on the transmission of *M. hyopneumoniae* under field conditions. Vet J 188:48–52
46. Pieters M, Daniels J, Rovira A (2017) Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet Microbiol 203:103–109
47. Chae C, Gomes-Neto JC, Segalés J, Sibila M (2020) Diversity of *Mycoplasma hyopneumoniae* strains. In: Book Mycoplasmas in Swine. Chapter 8. Editors: Maes D, Sibila M, Pieters M. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp. 147–161
48. Djordjevic SP, Eamens GJ, Romalis LF, Nicholls PJ, Taylor V, Chin J (1997) Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing

a denatured membrane antigen pool and adjuvant. Aust Vet J 75:504–511
49. Thacker EL, Thacker BJ, Boettcher TB, Jayappa H (1998) Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. J Swine Health Prod 6:107–112
50. Thacker EL, Thacker BJ, Kuhn M, Hawkins PA, Waters WR (2000) Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am J Vet Res 61:1384–1389
51. Sarradell J, Andrada M, Ramirez AS, Fernandez A, Gomez-Villamandos JC, Jover A, Lorenzo H, Herraez P, Rodriguez F (2003) A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Vet Pathol 40:395–404
52. Woolley L, Fell S, Gonsalves J, Raymond B, Collins D, Kuit T, Walker M, Djordjevic S, Eamens G, Jenkins C (2014) Evaluation of recombinant *Mycoplasma hyopneumoniae* P97/P102 paralogs formulated with selected adjuvants as vaccines against mycoplasmal pneumonia in pigs. Vaccine 32:4333–4341
53. Marchioro S, Maes D, Flahou B, Pasmans F, Del Pozo SR, Vranckx K, Melkebeek V, Cox E, Wuyts N, Haesebrouck F (2013) Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine. Vaccine 31:1305–1311
54. Deeney AS, Maglennon GA, Chapat L, Crussard S, Jolivet E, Rycroft AN (2019) *Mycoplasma hyopneumoniae* evades phagocytic uptake by porcine alveolar macrophages in vitro. Vet Res 50:51
55. Borjigin L, Shimazu T, Katayama Y, Li M, Satoh T, Watanabe K, Kitazawa H, Roh S, Aso H, Katoh K, Uchida T, Suda Y, Nakajo SA, M, Suzuki K, (2016) Immunogenic properties of Landrace pigs selected for resistance to mycoplasma pneumonia of swine. Anim Sci J 87:321–329
56. Abbas A, Lichtman A, Pillai S (2016) Basic immunology: functions and disorders of the immune system (Abbas AK, Ed. 5th edition). St. Louis, Missouri, USA: Elsevier
57. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, Goldman SJ, Dunussi-Joannopoulos K, Williams CM, Wright JF (2007) An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol 179:7791–7799
58. Jaffar Z, Ferrini ME, Herritt LA, Roberts K (2009) Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. J Immunol 182:4507–4511
59. Jones HP, Simecka JW (2003) T lymphocyte responses are critical determinants in the pathogenesis and resistance to mycoplasma respiratory disease. Front Biosci 8:930–945
60. Maes D, Boyen F, Dellagostin O, Shao G, Haesebrouck F (2020) Vaccines and vaccination against *Mycoplasma hyopneumoniae*. In: Book Mycoplasmas in Swine. Chapter 11. Editors: Dominiek Maes, Marina Sibila, Maria Pieters. ISBN 978-94-6379-796-2, Acco Publishers, Leuven Belgium. pp: 207–228
61. Park C, Jeong J, Choi K, Chae C (2016) Efficacy of a new bivalent vaccine of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Foster-aTM PCV MH) under experimental conditions. Vaccine 34:270–272
62. Feng Z, Wei Y, Li G, Lu X, Wan X, Pharr G, Wang Z, Kong M, Gan Y, Bai F, Liu M, Xiong Q, Wu X, Shao G (2013) Development and validation of an attenuated *Mycoplasma hyopneumoniae* aerosol vaccine. Vet Microbiol 167:417–424
63. Feng Z, Shao G, Liu M, Wu X, Zhou Y, Gan Y (2010) Immune responses to the attenuated *Mycoplasma hyopneumoniae* 168 strain vaccine by intrapulmonic immunization in piglets. Agr Sci China 9:423–431
64. Michiels A, Arsenakis I, Boyen F, Krejci R, Haesebrouck F, Maes D (2017) Efficacy of one dose vaccination against experimental infection with two *Mycoplasma hyopneumoniae* strains. BMC Vet Res 13:274
65. Vranckx K, Maes D, Villarreal I, Chiers K, Pasmans F, Haesebrouck F (2012) Vaccination reduces macrophage infiltration in bronchus-associated lymphoid tissue in pigs infected with a highly virulent *Mycoplasma hyopneumoniae* strain. BMC Vet Res 8:24
66. Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, Verbeke W, Viaene J, de Kruif A (1999) Effect of vaccination against *Mycoplasma hyopneumoniae* in pig herds with an all-in/all-out production system. Vaccine 17:1024–1034
67. Goodwin RF, Hodgson RG, Whittlestone P, Woodhams RL (1969) Some experiments relating to artificial immunity in enzootic pneumonia of pigs. J Hyg (Lond) 67:465–476
68. Martelli P, Saleri R, Cavalli V, De Angelis E, Ferrari L, Benetti M, Ferrarini G, Merialdi G, Borghetti P (2014) Systemic and local immune response in pigs intradermally and intramuscularly injected with inactivated *Mycoplasma hyopneumoniae* vaccines. Vet Microbiol 168:357–364
69. Maes D, Segalés J, Meyns T, Sibila M, Pieters M, Haesebrouck F (2008) Control of *Mycoplasma hyopneumoniae* infections in pigs. Vet Microbiol 126:297–309
70. Meyns T, Dewulf J, de Kruif A, Calus D, Haesebrouck F, Maes D (2006) Comparison of transmission of *Mycoplasma hyopneumoniae* in vaccinated and non-vaccinated populations. Vaccine 24:7081–7086
71. Sibila M, Nofrarias M, Lopez-Soria S, Segalés J, Valero O, Espinal A, Calsamiglia M (2007) Chronological study of *Mycoplasma hyopneumoniae* infection, seroconversion and associated lung lesions in vaccinated and non-vaccinated pigs. Vet Microbiol 122:97–107
72. Munyaka P, Blanc F, Estelle J, Lemonnier G, Leplat JJ, Rossignol MN, Jardet D, Plastow G, Billon Y, Willing B, Rogel-Gaillard C (2020) Discovery of predictors of *Mycoplasma hyopneumoniae* vaccine response efficiency in pigs: 16S rRNA gene fecal microbiota analysis. Microorganisms 8:1151
73. Pieters M, Fano E, Pijoan C, Dee S (2010) An experimental model to evaluate *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated sentinel pigs. Can J Vet Res 74:157–160
74. Betlach AM, Fano E, VanderWaal K, Pieters M (2021) Effect of multiple vaccinations on transmission and degree of *Mycoplasma hyopneumoniae* infection in gilts. Vaccine 39:767–774
75. Fagan P, Djordjevic S, Chin J, Eamens G, Walker M (1997) Oral immunization of mice with attenuated *Salmonella typhimurium* aroA expressing a recombinant *Mycoplasma hyopneumoniae* antigen (NrdF). Infect Immun 65:2502–2507
76. Chen AY, Fry S, Forbes-Faulkner J, Daggard G, Mukkur T (2006) Evaluation of the immunogenicity of the P97R1 adhesin of *Mycoplasma hyopneumoniae* as a mucosal vaccine in mice. J Med Microbiol 55:923–929
77. Chen AY, Fry S, Forbes-Faulkner J, Daggard G, Mukkur T (2006) Comparative immunogenicity of *M. hyopneumoniae* NrdF encoded in different expression systems delivered orally via attenuated *S* Typhimurium aroA in mice. Vet Microbiol 114:252–259
78. Okamba F, Moreau E, Cheikh Saad B, Gagnon C, Massie B, Arella M (2007) Immune responses induced by replication-defective adenovirus expressing the C-terminal portion of the *Mycoplasma hyopneumoniae* P97 adhesin. Clin Vaccine Immunol 14:767–774
79. Zou H, Liu X, Ma F, Chen P, Zhou R, He Q (2011) Attenuated *Actinobacillus pleuropneumoniae* as a bacterial vector for expression of *Mycoplasma hyopneumoniae* P36 gene. J Gene Med 13:221–229
80. Roques E, Girard A, Gagnon C, Archambault D (2013) Antibody responses induced in mice immunized with recombinant adenovectors expressing chimeric proteins of various porcine pathogens. Vaccine 31:2698–2704
81. Wang Y, Wang J, Zhou M, Liu P, Zhang E, Li Y, Lin J, Feng Z (2019) Yand Q (2019) Mucosal and systemic immune responses induced by intranasal immunization of recombinant *Bacillus subtilis* expressing the P97R1, P46 antigens of *Mycoplasma hyopneumoniae*. Biosci Rep. 39:BSR20191126
82. Menon S, Wannemuehler M, Mahairas G, Minion FC (2002) Mycobacterial ESAT-6 protein enhances mouse IFN-γ responses to *Mycoplasma hyopneumoniae* P71 protein. J Interf Cytok Res 22:807–813
83. Conceição F, Moreira A, Dellagostin O (2006) A recombinant chimera composed of R1 repeat region of *Mycoplasma hyopneumoniae* P97 adhesin with *Escherichia coli* heat-labile enterotoxin B subunit elicits immune response in mice. Vaccine 24:5734–5743
84. Barate A, Cho Y, Truon Q, Hahn T (2014) Immunogenicity of IMS 1113 plus soluble subunit and chimeric proteins containing *Mycoplasma hyopneumoniae* P97 C-terminal repeat regions. FEMS Microbiol Lett 352:213–220
85. Virginio V, Bandeira N, Leal F, Lancelotti M, Zaha A, Ferreira H (2017) Assessment of the adjuvant activity of mesoporous silica nanoparticles in recombinant *Mycoplasma hyopneumoniae* antigen vaccines. Heliyon 3:e00225

86. De Oliveira NR, Jorge S, Gomez CK, Rizzi C, Dias Pacce V, Collares F, Monte GL, Dellagostin O (2017) A novel chimeric protein composed of recombinant *Mycoplasma hyopneumoniae* antigens as a vaccine candidate evaluated in mice. Vet Microbiol 201:146–153
87. Chen Y, Wang S, Yang W, Chen Y, Lin H, Shiuan D (2003) Expression and immunogenicity of *Mycoplasma hyopneumoniae* heat shock protein antigen p42 by DNA vaccination. Infect Immun 71:1155–1160
88. Minion FC, Menon S, Mahairas G, Wannemuehler M (2003) Enhanced murine antigen-specific gamma interferon and immunoglobulin G2a responses by using mycobacterial ESAT-6 sequences in DNA vaccines. Infect Immun 71:2239–2243
89. Chen AY, Fry S, Daggard G, Mukkur K (2008) Evaluation of immune response to recombinant potential protective antigens of *Mycoplasma hyopneumoniae* delivered as cocktail DNA and/or recombinant protein vaccines in mice. Vaccine 26:372–378
90. Galli V, Simionatto S, Marchioro S, Fisch A, Gomes C, Conceição F, Dellagostin O (2012) Immunisation of mice with *Mycoplasma hyopneumoniae* antigens P37, P42, P46 and P95 delivered as recombinant subunit or DNA vaccines. Vaccine 31:135–140
91. Virginio V, Gonschoroski T, Paes J, Schuck D, Zaha A, Ferreira H (2014) Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential for use in vaccination against porcine enzootic pneumonia. Vaccine 44:5832–5838
92. Stevens T, Bossie A, Sanders V, Fernandez-Botran R, Coffman R, Mosmann T, Vitetta E (1998) Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 334:255–258
93. Fairbairn L, Kapetanovic R, Sester DP, Hume DA (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol 89:855–871
94. Fagan P, Walker M, Chin J, Eamens G, Djordjevic S (2001) Oral immunization of swine with attenuated *Salmonella typhimurium* aroA SL3261 expressing a recombinant antigen of *Mycoplasma hyopneumoniae* (NrdF) primes the immune system for a NrdF specific secretory IgA response in the lungs. Microb Pathog 30:101–110
95. Shimoji Y, Oishi E, Muneta Y, Nosaka H, Mori Y (2003) Vaccine efficacy of the attenuated *Erysipelothrix rhusiopathiae* YS-19 expressing a recombinant protein of *Mycoplasma hyopneumoniae* P97 adhesin against mycoplasmal pneumonia of swine. Vaccine 21:532–537
96. Ogawa Y, Oishi E, Muneta Y, Sano A, Hikono H, Shibahara T, Yagi Y, Shimoji Y (2009) Oral vaccination against mycoplasmal pneumonia of swine using a live *Erysipelothrix rhusiopathiae* vaccine strain as a vector. Vaccine 27:4543–4550
97. Okamba F, Arella M, Music N, Jia J, Gottschalk M, Gagnon C (2010) Potential use of a recombinant replication-defective adenovirus vector carrying the C-terminal portion of the P97 adhesin protein as a vaccine against *Mycoplasma hyopneumoniae* in swine. Vaccine 28:4802–4809
98. King K, Faulds D, Rosey E, Yancey R Jr (1997) Characterization of the gene encoding Mhp1 from *Mycoplasma hyopneumoniae* and examination of Mhp1's vaccine potential. Vaccine 15:25–35
99. Okada M, Asai T, Ono M, Sakano T, Sato S (2000) Protective effect of vaccination with culture supernate of *M. hyopneumoniae* against experimental infection in pigs. J Vet Med B 47:527–533
100. Okada M, Asai T, Ono M, Sakano T, Sato S (2000) Cytological and immunological changes in bronchoalveolar lavage fluid and histological observation of lung lesions in pigs immunized with *Mycoplasma hyopneumoniae* inactivated vaccine prepared from broth culture supernate. Vaccine 18:2825–2831
101. Chen J, Liao C, Mao S, Weng C (2001) A recombinant chimera composed of repeat region RR1 of *Mycoplasma hyopneumoniae* adhesin with Pseudomonas exotoxin: in vivo evaluation of specific IgG response in mice and pigs. Vet Microbiol 80:347–357
102. Lee SH, Lee S, Chae C, Ryu D-Y (2014) A recombinant chimera comprising the R1 and R2 repeat regions of *M. hyopneumoniae* P97 and the N-terminal region of *A. pleuropneumoniae* ApxIII elicits immune responses. BMC Vet Res. 10:43
103. Lin J, Pan M, Liao C, Weng C (2002) In vivo and in vitro comparisons of spray-drying and solvent-evaporation preparation of microencapsulated *Mycoplasma hyopneumoniae* for use as an orally administered vaccine for pigs. Am J Vet Res 63:1118–1123
104. Lin J, Weng C, Liao C, Yeh K, Pan M (2003) Protective effects of oral microencapsulated *Mycoplasma hyopneumoniae* vaccine prepared by co-spray drying method. J Vet Med Sci 65:69–74
105. Matthijs A, Auray G, Jakob V, García-Nicolás O, Braun R, Keller I, Bruggman R, Devriendt B, Boyen F, Guzman C, Michiels A, Haesebrouck F, Collin N, Barnier-Quer C, Maes D, Summerfield A (2019) System immunology characterization of novel vaccine formulations for *Mycoplasma hyopneumoniae* bacterins. Front Immunol 10:1087
106. Matthijs A, Auray G, Boyen F, Michiels A, Garcia-Nicolas O, Barut GT, Barut G, Barnier-Quer C, Jakob V, Collin N, Devriendt B, Summerfield A, Haesebrouck F, Maes D (2019) Efficacy of three innovative bacterin vaccines against experimental infection with *Mycoplasma hyopneumoniae*. Vet Res 50:91
107. Meens J, Bolotin V, Frank R, Böhmer J, Gerlach GF (2010) Characterization of a highly immunogenic *Mycoplasma hyopneumoniae* lipoprotein Mhp366 identified by peptide-spot array. Vet Microbiol 142:293–302
108. Pendarvis K, Padula MP, Tacchi JL, Petersen AC, Djordjevic SP, Burgess SC, Minion FC (2014) Proteogenomic mapping of *Mycoplasma hyopneumoniae* virulent strain 232. BMC Genomics 15:576
109. Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148–158
110. Aoshi T (2017) Modes of action for mucosal vaccine adjuvants. Viral Immunol 30:463–470
111. Figueras Gourgues S, Fano E, Alegre Sabaté A, López Grasa E, Hernández Caravaca I, García Vázquez FA, Rodríguez Vega V, Garcia-Morante B (2020) Assessment of nebulization technology for gilt exposure to *Mycoplasma hyopneumoniae* as an acclimation strategy. J Swine Health Prod 28:294–301
112. Wilson HL, Obradovic MR (2015) Evidence for a common mucosal immune system in the pig. Mol Immunol 66:22–34
113. Fu T, Ulmer JB, Caufield MJ, Deck RR, Friedman A, Wang S, Liu X, Donnelly JJ, Liu MA (1997) Priming of cytotoxic T lymphocytes by DNA vaccines. Requirement for professional antigen presenting cells and evidences for antigen transfer from myocytes. Mol Med 3:362–371
114. Bastola R, Noh G, Keum T, Bashyal S, Seo JE, Choi J, Oh Y, Cho Y, Lee S (2017) Vaccine adjuvants: smart components to boost the immune system. Arch Pharm Res 40:1238–1248
115. Villarreal I, Maes D, Meyns T, Gebruers F, Calus D, Pasmans F, Haesebrouck F (2009) Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a highly virulent *M. hyopneumoniae* isolate. Vaccine 27:1875–1879

Publisher's Note