Evaluation of hydrophobic chitosan-based particulate formulations of porcine reproductive and respiratory syndrome virus vaccine candidate T cell antigens

Helen Mokhtar[a,b], Lucia Biffar[a], Satyanarayana Somavarapu[c], Jean-Pierre Frossard[a], Sarah McGowan[a], Miriam Pedrera[a,2], Rebecca Strong[a], Jane C. Edwards[a], Margarita Garcia-Durán[d], Maria Jose Rodriguez[d], Graham R. Stewart[b], Falko Steinbach[a,b], Simon P. Graham[a,b,1]

[a]Virology Department, Animal Plant Health Agency, Addlestone, KT15 3NB, United Kingdom. [b]Faculty of Health and Medical Sciences, University of Surrey, Guildford, GU2 7XH, United Kingdom. [c]School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX, United Kingdom. [d]Ingenasa, C/ Hermanos García Noblejas, 41, 2º 28037 Madrid, Spain.

[1]Corresponding author and present address: Dr Simon Graham, The Pirbright Institute, Ash Road, Pirbright, Woking, GU24 0NF, United Kingdom. Tel: +44 (0)1483 232 441. E-mail: simon.graham@pirbright.ac.uk. [2]Present address: The Pirbright Institute, Ash Road, Pirbright, Woking, GU24 0NF, United Kingdom.

Abstract

PRRS control is hampered by the inadequacies of existing vaccines to combat the extreme diversity of circulating viruses. Since immune clearance of PRRSV infection may not be dependent on the development of neutralising antibodies and the identification of broadly-neutralising antibody epitopes have proven elusive we hypothesised that conserved T cell antigens represent potential candidates for development of a novel PRRS vaccine. Previously we had identified the M and NSP5 proteins as well-conserved targets of polyfunctional CD8 and CD4 T cells. To assess their vaccine potential, peptides representing M and NSP5 were encapsulated in hydrophobically-modified chitosan particles adjuvanted by incorporation of a synthetic multi-TLR2/TLR7 agonist and coated with a model B cell PRRSV antigen. For comparison, empty particles and adjuvanted particles encapsulating inactivated PRRSV-1

were prepared. Vaccination with the particulate formulations induced antigen-specific antibody responses, which were most pronounced following booster immunisation. M and NSP5-specific CD4, but not CD8, T cell IFN-γ reactivity was measurable following the booster immunisation in a proportion of animals vaccinated with peptide-loaded particles. Upon challenge, CD4 and CD8 T cell reactivity was detected in all groups, with the greatest responses being detected in the peptide vaccinated group but with limited evidence of an enhanced control of viraemia. Analysis of the lungs during the resolution of infection showed significant M/NSP5 specific IFN-γ responses from CD8 rather than CD4 T cells. Vaccine primed CD8 T cell responses may therefore be required for protection and future work should focus on enhancing the cross-presentation M/NSP5 to CD8 T cells.

Keywords: porcine reproductive and respiratory syndrome virus, chitosan particles, vaccine formulation, T cell,

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important pig diseases worldwide. The causative PRRS virus (PRRSV) is rapidly evolving and there is an urgent need for the development of safer and more efficacious vaccines to improve PRRS control. Efforts to develop improved vaccines have focused primarily on the structural envelope glycoproteins but these have met with limited success and thus alternative approaches are required (Murtaugh and Genzow, 2011; Renukaradhya et al., 2015). While the immunological mechanisms underlying protection against PRRSV remain to be fully defined, there is evidence to suggest that cell-mediated immune responses play an important role (Murtaugh and Genzow, 2011; Zuckermann et al., 2007). Indeed, T cells are crucial to the control of many viruses through cytolysis of infected cells and cytokine secretion. Since clearance of PRRSV infection may not be dependent on neutralising antibodies, we

hypothesised that conserved PRRSV T cell antigens should be considered as an integral component of any next-generation PRRSV vaccine. Through proteome-wide peptide library screening, we identified both the structural matrix (M) protein and the non-structural protein 5 (NSP5) from PRRSV genotype 1 (PRRSV-1) as well conserved targets of T cell immunity (Mokhtar et al., 2016), which warranted further evaluation of their vaccine potential.

Amongst the range of vaccine delivery systems being developed to induce T cell mediated immunity to defined antigens, one strategy which possesses a number of desirable features is the use of nano- or micro-particles to deliver vaccine antigen to dendritic cells (DCs). Both antigens in protein or plasmid DNA form can be encapsulated inside or coated to the surface of biodegradable particles which allows for sustained release after administration, increasing the bioavailability and decreasing the degradation (Bivas-Benita et al., 2004; Shen et al., 2006). The efficient internalisation of the particle is a distinct advantage and if taken up by dendritic cell (DC) allows the cargo of antigen to be potentially processed via the MHC class I or II presentation pathways (Hirosue et al., 2010). In addition, particles can carry molecular adjuvants thus delivering the appropriate stimulatory signals together with the antigen to individual DC. Particulate vaccines have been evaluated in the context of PRRSV, with killed PRRSV encapsulated inside poly(lactic, glycolic acid) (PLGA) particles and administered intranasally to pigs resulting in enhanced antibody and T cell responses that translated to cross-protective immunity (Binjawadagi et al., 2014a; Binjawadagi et al., 2014b; Dwivedi et al., 2013). Amongst the available polymers used to produce particles, chitosan has a number of attractive features for vaccine delivery. Chitosan and its derivatives are abundantly expressed biological polysaccharides which readily form particles (Jayakumar et al., 2010). Chitosan may offer an inherent adjuvant effect through binding the innate immune sensor, Toll-like receptor 4 (TLR4), and has been shown to bind specifically to mannose receptors expressed on DCs (Villiers et al., 2009). This study therefore evaluated the immunogenicity

of chitosan particles loaded with M and NSP5 antigens in comparison to inactivated PRRSV-1. Since it has been shown that targeting multiple TLRs augments vaccine immunogenicity and efficacy (Kasturi et al., 2011; Querec et al., 2006), particles were adjuvanted by incorporation of the synthetic multi-TLR2/TLR7 agonist Adilipoline™.

Materials and methods

Ethics statement

All work was approved by the APHA Ethics Committee and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Project Licence numbers PPL 70/7057 and 70/7209.

PRRS viruses, peptides and proteins

The PRRSV-1 subtype 1 strains Olot/91 and 215-06 were propagated and titrated in MARC-145 cells and porcine alveolar macrophages, respectively (Mokhtar et al., 2014; Morgan et al., 2013). For the vaccine formulation, PRRSV-1 Olot/91 was grown in serum free RPMI-1640 medium, semi-purified and concentrated using a 300kDa MicroKros Filter Module (Spectrum Labs, Breda, The Netherlands) and inactivated by incubation with a 1:1000 dilution of β-propriolactone (Sigma-Aldrich, Poole, UK) at 37°C for 2 hours with continuous shaking. Inactivation was confirmed by virus titration. A recombinant fusion protein, EM4, consisting of the GP5 ectodomain (MSSTYQYIYNLTICELNGTDWLSNHA, that corresponds to amino acids 37-61 of GP5, except the terminal methionine and alanine residues that were added for cloning purposes) fused directly to the C-terminal region of the M protein (MDAHHVKSAAGLHSIPASGNRAYAVRKPGLTSVNGTLVPGLRSL, corresponding to amino acids 112-151 of M protein except the two first residues that were included for cloning purposes) from PRRSV-1 Olot/91 was expressed by a recombinant baculovirus in Sf9 cells. EM4 was purified from infected cell lysates by Ni^{2+} affinity

chromatography, dialysed and detected by western blotting. Overlapping 20mer peptides, offset by 10 amino acids, encompassing the entire M and NSP5 proteins from PRRSV-1 Olot/91 strain were synthesised (Mimotopes, Heswall, UK) and reconstituted in sterile DMSO (Sigma-Aldrich, Poole, UK).

Particulate PRRSV vaccine formulations

Three chitosan-based particulate formulations were prepared: (1) Inactivated PRRSV-1 Olot/91 loaded particles (Virus-P), (2) M and NSP5 peptide loaded and EM4-coated particles (Peptide-P) and (3) control empty particles (Empty-P). For each vaccine dose of particles, 50mg of octanyl-chitosan (chitosan from Heppe Medical Chitosan GmbH, Halle, Germany) dissolved in chloroform was mixed with the following: Virus-P - 50µg Adilipoline™ (Invivogen, Source Bioscience, Nottingham, UK), $6x10^7$ $TCID_{50}$ equivalent inactivated PRRSV-1 Olot/91; Peptide-P - 50µg Adilipoline, 100µg of each 20mer M and NSP5 peptides; and Empty-P - sterile deionised water. 15% PVA was added to each preparation and homogenised for 1 minute followed by probe sonication for 2 minutes (amplitude 17 microns). The mixtures were then added drop-wise to 1% PVA whilst homogenising for 4 minutes. Following removal of solvent by evaporation, the following were adsorbed onto the surface of the particles by adding drop-wise whilst on a magnetic stirring block: Virus-P - 50µg Adilipoline/dose; Peptide-P - 50µg Adilipoline and 200µg EM4 protein/dose and Empty-P were left untreated. All vaccine formulations were diluted to 4ml per dose with sterile water. The surface charge of particles was measured using a Zeta Sizer 3000 (Malvern Instruments, Malvern, UK) and particle morphology and size assessed using scanning electron microscopy (Walters et al., 2015). To assess loading and coating efficiencies aliquots of each formulation were centrifuged at 14000 rpm for 5 min and supernatants were harvested and stored at -80°C until analysed. ELISA and biochemical assay analyses of particle-free supernatants were conducted to determine the efficiency of virus (ELISA) or

peptide (fluorometric *o*-phthalaldehyde assay) encapsulation and coating with EM4 protein (ELISA). Absorption of EM4 to particle surfaces was additionally assessed by flow cytometric staining of particles (Walters et al., 2015). Further details of these assays are provided in Supplementary Materials.

In vitro assessment of the innate immunostimulatory properties of particulate PRRSV vaccine formulations

To assess stimulation of monocytes and DCs with particles, heparinised blood was collected from PRRSV antibody negative Large White/Landrace cross-bred pigs 6 months of age. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Franzoni et al., 2013a). Monocytes and dendritic cells (DCs) were enriched separately from PBMC by magnetic bead based sorting (Franzoni et al., 2014). In brief, PBMC were incubated with human CD14 microbeads (Miltenyi Biotec, Bisley, UK), and applied to a LD column (Miltenyi Biotec). The negative flow through ($CD14^-$) fraction were sequentially stained with 10µg/10^8 cells of mouse anti-porcine CD172a mAb (clone 74-22-15A, Washington State University Monoclonal Antibody Center (WSUMAC), Pullman, USA) and mouse IgG microbeads (Miltenyi Biotec). $CD172a^+$ cells were enriched using an LS column (Miltenyi Biotec). To obtain a purer monocyte population the $CD14^+$ fraction was also enriched using an LS column. $CD172a^+$ (DC) and $CD14^+$ (monocytes) fractions were eluted and adjusted to $4x10^6$ cells/ml in RPMI-1640 medium with 10% FBS (cRPMI), which was further supplemented for DC cultures with 20ng/ml of recombinant porcine IL-3 (Franzoni et al., 2014). Cells were seeded 50µl/well in 96 well round bottom plates and stimulated with titrations of Virus-, Peptide- or Empty-P. Unstimulated and Adilipoline (10µg/ml) stimulated cells were included as negative and positive controls respectively. After 18 hours culture at 37^{o}C, cytokines were quantified in cell-free culture supernatants using commercial ELISA

kits for porcine IL-8, IL-10 and IL-12 (all R&D Systems) and type I IFN bioactivity reporter gene assay (Franzoni et al., 2014).

Immunisation of pigs with particulate PRRSV vaccine formulations and challenge with PRRSV

Eighteen PRRSV antibody negative Large White/Landrace male piglets 8 weeks of age were randomly assigned to three groups (n=6) which were vaccinated subcutaneously behind each ear with 2ml of either the Virus-P, Peptide-P or Empty-P formulations. An identical booster vaccination was given on day 21 post-primary vaccination. Animals were challenged intranasally on day 42 post-primary vaccination with 10^5 $TCID_{50}$ of PRRSV-1 215-06 and monitored for clinical scores and rectal temperatures until the end of the study, day 63 post-primary vaccination (day 21 post-challenge) (Morgan et al., 2013). Heparinised blood and serum samples were collected at weekly intervals during the study to analyse immune responses. RNA was isolated from EDTA blood samples collected every 2-3 days from day -2 post-challenge using the QIAamp RNA Blood Mini Kit (QIAGEN, Crawley UK) and PRRSV RNA measured using the QuantiTect® Probe RT-PCR kit (QIAGEN)((Frossard et al., 2012). PRRSV genome copy numbers were interpolated using a standard PRRSV-1 nucleocapsid gene (ORF7) RNA prepared from PRRSV-1 215-06 (Morgan et al., 2013).

Measurement of PRRSV-specific antibody responses

EM4 specific serum antibody responses were analysed by ELISA. ELISA plates (MAXISorb, Nunc) were coated overnight at 4°C with 1µg/ml recombinant EM4 protein in carbonate-bicarbonate buffer. After washing and blocking in BSA, serum, diluted 1 in 100, was added for 1h at room temperature (RT). After washing, goat anti-porcine IgG-HRP antibody (Jackson ImmunoResearch, Stratech Scientific Limited, Newmarket, UK) diluted 1/20,000 was added for 1h at RT. Plates were washed and developed by addition of TMB substrate

(Sigma). PRRSV N protein-specific antibody responses were analysed using the INgezim PRRS 2.0 ELISA (Ingenasa). PRRSV neutralizing antibody titres in serum samples were determined as described (Weesendorp et al., 2013) with minor modifications. Serial 2-fold dilutions of heat-inactivated sera were incubated with 400 $TCID_{50}$ of PRRSV-1 Olot/91 for 1hr at 37^{o}C before addition of MARC-145 cells ($5x10^3$ cells/well). After 72 hours incubation, infection was assessed by immunoperoxidase staining and antibody titres calculated as log_2 of the reciprocal serum dilution that fully neutralized viral replication in 50% of the wells (ND_{50}).

Measurement of antigen-specific T cell responses

PBMC were suspended in cRPMI at a density of $1x10^7$/ml and seeded 100µl/well in 96 well round bottom plates (Costar, Fisher Scientific) and stimulated with 100µl PRRSV-1 Olot/91 at a multiplicity of infection (MOI) of 0.1, M and NSP5 peptide pools with each constituent peptide at 1µg/ml. Negative controls were PBMC incubated in cRPMI alone or with mock-virus supernatants. Virus and peptide stimulated PBMC were incubated before the addition and further incubation of brefeldin A (0.1µl/well) (GolgiPlug, BD Biosciences) as described (Franzoni et al., 2013b). After incubation, PBMC were stained with Zombie Near Infra-Red Fixable Viability Kit (Biolegend, London, UK), CD4-PerCP-Cy5.5 (clone 74-12-4, BD Biosciences) and CD8α-PE (clone 76-2-11, BD Biosciences) mAbs, fixed/permeabilized in CytoFix/CytoPerm solution (BD Biosciences), stained with IFN-γ-Alexa Fluor 647 (clone CC302, AbD Serotec) and TNF-α-Brilliant Violet 421 (clone MAb11, Biolegend) mAbs or the respective isotype controls (Alexa Fluor 647 mouse IgG1 isotype control, AbD Serotec, and Brilliant Violet 421 mouse IgG1 isotype control, Biolegend) and analysed using a MACSQuant Analyzer flow cytometer (Franzoni et al., 2013a).

On day 21 post-challenge, animals were euthanized and bronchoalveolar lavage fluid (BALF) collected (Morgan et al., 2013). BALF cellularity was analysed directly using a volumetric

MACSQuant Analyzer flow cytometer. The T cell composition of BALF was determined by staining with CD3-FITC (clone BB23-8E6-8C8; BD Biosciences), CD4-PerCP-Cy5.5 (clone 74-12-4) and CD8α-PE (clone 76-2-11) mAbs for 30 minutes at 4ºC. Cells were washed twice in PBS and analysed by flow cytometry. To assess antigen-specific T cell responses, mononuclear cells were isolated by density gradient centrifugation, stimulated with PRRSV-1 Olot/91 or a pool of M/NSP5 peptides and IFN-γ and TNF-α expression by CD4 and CD8 T cells analysed as described above.

Statistical analysis

GraphPad Prism 6.01 (GraphPad Software, La Jolla, USA) was used for graphical and statistical analysis of data sets. Analysis of variance (ANOVA) was employed to analyse fixed effects on different traits with various post-hoc tests as detailed in figure legends**.** A p value < 0.05 was considered statistically significant.

Results

Design and quality control of particulate vaccines

Adilipoline adjuvanted chitosan-based particulate formulations of PRRSV antigens were prepared as illustrated in Figure 1A. The first formulation was made by encapsulating inactivated PRRSV-1 Olot/91 (Virus-P). Inactivated virus served as a crude and diverse mixture of antigens, which may be recognised by both B and T cells. This formulation was adjuvanted by encapsulation of and coating with Adilipoline. The second formulation was similarly adjuvanted but particles encapsulated synthetic peptides representing the M and NSP5 proteins, as T cell antigens, and were coated with a recombinant M/GP5 fusion protein fragment (EM4) possessing a non-neutralising antibody epitope, which served as a model B cell antigen (Peptide-P). The third formulation, which served as the negative control, consisted of empty chitosan particles (Empty-P). Scanning electron microscopy showed

particles with a spherical morphology and a typical diameter less than 5µm (Figure 1B). The Virus-P tended to be larger and to collapse more easily, possibly due to the volume of antigen encapsulated. Zeta potential measurements showed the Virus-P to be negatively charged whereas the Peptide-P and Empty-P were positively charged, which most likely reflected their differing composition (Figure 1C).

To assess the efficiency of virus or peptide encapsulation and coating with EM4 protein, antigen was quantified in particle-free supernatants (Supplementary Figure S1). Inactivated PRRSV Olot/91 and EM4 were assessed using antigen-capture ELISAs and the encapsulation efficiencies were calculated to be over 99% efficient. The adsorption of EM4 protein onto the surface of ~80% of Peptide-P was additionally demonstrated by flow cytometry (Supplementary Figure S2). Given the high efficiency of EM4-coating, a fluorometric biochemical assay was conducted to assess the encapsulation of M and NSP5 peptides which were be estimated to be at >98% efficient (Supplementary Figure S1). Assessment of Adilipoline incorporation was not undertaken as it was not readily amenable to assessment by ELISA or other means e.g. HPLC.

The particles were screened *in vitro* to assess the cytokine response profiles of blood DCs and monocytes (Figure 2). Cultures of enriched blood DCs, which included both plasmacytoid and conventional DC subsets (Franzoni et al., 2014), and monocytes were cultured in the presence of titrations of particle formulations, which for Peptide- and Virus-P were additionally tested with (‘washed’) and without (‘unwashed’) pre-washing to potentially remove any unbound Adilipoline. Unstimulated and soluble Adilipoline stimulation conditions were included as negative and positive controls, respectively. Statistically significant IL-8 responses from DCs and monocytes were induced by both the washed and unwashed adjuvanted particles. Interestingly, Empty-P also induced an IL-8 response from monocytes, albeit of a lesser magnitude than the adjuvanted particles, but this was not

detectable in DC cultures. No IL-10 was detected in supernatants from DC cultures and only Virus-P induced a statistically significant monocyte IL-10 response, suggestive that this was virus- rather than Adilipoline-specific. Whilst not detected in monocyte cultures, statistically significant IL-12 and type I IFN responses were detected in DC cultures following stimulation with both Virus- and Peptide-P, irrespective of washing, suggesting that particle-associated Adilipoline was inducing these responses.

Assessment of immune responses and protection following experimental vaccination in pigs

Groups of pigs (n=6) were vaccinated twice with each of the particulate formulations (Virus-P, Peptide-P and Empty-P) with a three week interval between prime and booster inoculations. After a further three weeks, all animals were challenged with a UK field strain of PRRSV-1 (215-06) and assessed for protection. Over the duration of the experiment, the induction of antigen-specific antibody and T cell responses were monitored and protection assessed by measuring viraemia by quantitative RT-PCR.

Assessment of PRRSV-specific antibody responses using a commercial N protein-based ELISA showed that after the booster immunization, significant levels of specific antibodies were detected in the Virus-P immunised group (Figure 3A). These responses were rapidly boosted by challenge infection and were significantly greater than the Peptide- and Empty-P groups on days 7 and 14 post-challenge). Significant EM4-specific antibody responses were detected in the Peptide-P immunised group from day 28 (7 days post booster immunisation) which remained elevated for the duration of the study (Figure 3B). As expected for this subdominant epitope there was little evidence of further boosting of this response upon the challenge infection and levels remained low in the Virus- and Empty-P groups. Assessment of virus-neutralising antibodies in serum samples revealed only two pigs from the Virus-P immunised group with low ND_{50} titres of 4 (pig 12) and 11 (pig 15) (data not shown).

Following *in vitro* stimulation of PBMC with PRRSV-1 Olot/91, M and NSP5 peptides, IFN-γ and TNF-α co-expression by CD4 and CD8 T cells was assessed by flow cytometry. Single cytokine producing cells were also assessed however very few single cytokine expressing cells were identified and therefore only data for dual-cytokine expressing cells are presented (Figure 4). Post-vaccination, the only significant responses detected were M and NSP5-specific CD4 T cells responses from 3/6 Peptide-P immunised pigs and virus-specific CD4 T cells responses from an individual animal in each of the Peptide-P and Virus-P immunised groups. Significant responses were detected in all groups post-challenge: the majority of pigs in each group mounted both CD4 and CD8 T cell responses to M peptides and PRRSV. NSP5-specific CD4 T cell responses were detected in one of the Virus-P immunised pigs and four of the Peptide-P immunised pigs. 2/6 Virus-P, 3/6 Peptide-P and 4/6 Empty-P immunised pigs displayed significant NSP-5 specific CD8 T cell responses.

Three weeks after the booster immunisation, all pigs were challenged intranasally with PRRSV-1 215-06 and the course of RNAemia assessed by RT-qPCR (Figure 5). PRRSV RNA levels peaked in animals regardless of vaccination group on day 7 post-challenge with the highest levels detected in the Peptide-P immunised group. Levels declined thereafter with the lowest levels detected in the Peptide-P group albeit without statistical significance.

Upon termination of the experiment, day 21 post-challenge, lungs were lavaged post-mortem. The BALF cellularity and composition was determined, and T cell responses measured following stimulation with PRRSV or a pool of M/NSP5 peptides (Figure 6). For technical reasons it was not possible to collect BALF from all animals. The results showed that for all animals irrespective of vaccine group there was a significantly greater number of CD8 compared to CD4 T cells within the lavage fluid. Assessment of antigen-specific responses showed that again for all animals there was a statistically significantly higher frequency of

PRRSV and M/NSP5 specific CD8 T cells compared to CD4. The frequency of antigen-specific CD8 T cells in BALF was much higher than had been detected in blood (Figure 5).

Discussion

The results of this study have shown that hydrophobically-modified chitosan may be used to efficiently generate particulate formulations of either whole PRRSV or defined PRRSV antigens. Further enhancement of the immunostimulatory nature of these particles by co-formulation with the multi-PRR ligand Adilipoline was demonstrated *in vitro*. However, despite the encouraging *in vitro* data, vaccination with these formulations induced antibody responses but failed to induce strong antigen-specific T cell responses, which are believed to be required for the effective control of PRRSV infection in the absence of neutralising antibodies (Morgan et al., 2013; Murtaugh and Genzow, 2011; Weesendorp et al., 2013; Zuckermann et al., 2007). It is worthy to note that the Peptide-P formulation appeared to prime at least in half the animals CD4 as opposed to CD8 T cell responses. The data from the lung lavages following the resolution of the PRRSV infection was striking and would further suggest that it was a lack of CD8 T cell response that may underlie the poor vaccine efficacy observed. The vaccine formulations did result in cross-presentation of antigen to memory CD8 T cells *in vitro*, albeit with a lower efficiency than to CD4 T cells (data not shown). Several factors may underlie the poor T cell immunogenicity of these particles including the choice of polymer, the size of the particles and the quantity of antigen/adjuvant loaded. Chitosan was selected as the polymer core of the particulate vaccines due to its inherent immunostimulatory properties (Highton et al., 2015; Zaharoff et al., 2007; Zhu et al., 2007), which is thought to be mediated through engagement of TLR4 and mannose receptors on antigen-presenting cells (Villiers et al., 2009). Rather unexpectedly, the results of the *in vitro* evaluation of our chitosan particles showed that non-adjuvanted particles failed to induce cytokine responses from porcine DCs and elicited only an IL-8 response from monocytes.

The formulated particles in the present study were in the low micron range, which is at the upper range of optimal particle size for phagocytosis by DCs and macrophages (He et al., 2010; Thiele et al., 2001). Whilst outside the scope of the present study, formulation conditions may be manipulated to create antigen encapsulated chitosan particles with discrete nominal sizes. A recent study of chitosan particles of 300nm, 1µm, and 3µm parameters showed that 1µm particles were optimal for phagocytosis by macrophages but uptake by DC was independent of particle size (Koppolu and Zaharoff, 2013). Interestingly the three particle sizes induced discrete DC cytokine response profiles and the 3µm particles were best at stimulating antigen-presentation and CD4 T cell responses; which was attributed to augmented antigen encapsulation efficiency (Koppolu and Zaharoff, 2013). In the present study, we indirectly assessed the formulated antigen cargo by quantifying antigen that remained in supernatants following formulation. Whilst these analyses suggested very high (>90%) encapsulation and coating efficiencies, in line with previous studies (Koppolu and Zaharoff, 2013; Li et al., 2008; Oliveira et al., 2012), assessment of entrapped antigen following chitinase digestion may have verified whether the antigens had maintained their stability upon encapsulation. Presumably due to Adilipoline engagement with TLR-7, adjuvanted formulations also induced significant IL-12 and IFN-α responses from DC, which are both reported to drive cross-presentation and induction of CD8 T cell responses (Janssen and Thacker, 2012; Joffre et al., 2012; Oh et al., 2011). The $XCR1^+$ conventional DC subset (cDC1) is highly efficient at cross-presenting exogenous antigen to $CD8^+$ T cells and mice lacking XCR1 or its ligand, are less able to cross-present antigen necessary for induction of $CD8^+$ T cell responses against viruses and intracellular bacteria (Fries, 2016; Shortman and Heath, 2010). Further work should therefore address how efficiently chitosan particles are taken up and processed by porcine cCD1, since enhancing the targeting and activation of this DC subset could be crucial to enhancing CD8 T cell induction *in vivo*.

345 Another factor which needs to be considered in the future is the route of inoculation. In
346 contrast to peripheral blood where both CD4 and CD8 T cell responses were detected, the
347 virus and M/NSP5 specific response in the lung were almost exclusively from CD8 T cells
348 and this was accompanied by a greater infiltrate of these cells compared to CD4 T cells.
349 These data support earlier observations (Samsom et al., 2000) and suggest that the CD8 T cell
350 population performs a critical role in the effector phase of the immune response in clearing
351 virus from the lungs. While the subcutaneous route ensured efficient delivery of the vaccine
352 *in vivo*, recent studies have shown that targeting DCs at mucosal sites is crucial to programme
353 mucosa-homing T cell responses (Mikhak et al., 2013; Sandoval et al., 2013). This is
354 supported by the recent reports that intranasal delivery of an adjuvanted PLGA based
355 nanoparticulate formulation of crude PRRSV antigens protected against heterologous
356 challenge infection (Binjawadagi et al., 2014a; Binjawadagi et al., 2014b). The inherent
357 mucoadhesive properties of hydrophobic chitosan (Patel et al., 2010) makes this a viable
358 proposal for investigating the induction of mucosal immunity which may be required for
359 protection against PRRSV and intranasal or aerosol immunisation should be considered in
360 future work.
361 Alternatives to particulate delivery should also be considered for induction of CD8 T cell
362 responses against M and NSP5. The use of viral vectors such as recombinant replication-
363 deficient adenovirus or pox virus vectors are the subject intense investigation for the
364 development of vaccines to T cell responses against a range of intracellular pathogens (Ertl,
365 2016; Sebastian and Gilbert, 2016). However, another viral vector system that warrants
366 consideration for induction of T cell immunity is the use of cytomegaloviruses (CMV). The
367 capacity of CMV-based vaccines to induce T cell responses against virulent pathogens
368 entering via mucosal sites as was most strikingly demonstrated by the prevention of lethal
369 simian immunodeficiency virus (SIV) infection in rhesus macaques following challenge of

animals vaccinated with rhesus CMV (RhCMV)-based vectors expressing SIV antigens (Hansen et al., 2011). Porcine CMV (PCMV) in pigs appears comparable to CMV infection in other species. Although PCMV remains poorly characterised in immunological terms compared to other CMVs (mouse, rhesus macaque and human), the CMV-specific immune response is remarkably conserved between different CMV species, strongly suggesting that PCMV will have similar immunological characteristics. The annotated PCMV genome was recently published (Gu et al., 2014), which makes the design and construction of PCMV-based vectors expressing PRRSV M and NSP5 possible for the first time.

In conclusion, this study has provided further data to support PRRSV M and NSP5 as vaccine candidate T cell antigens. The biomimetic particulate formulations showed promising initial results and whilst immunogenicity may be readily improved by virtue of being a highly customisable mode of formulation, delivery of M and NSP5 by live viral vectors should also be explored.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Formulation of PRRSV antigens in adjuvanted hydrophobic chitosan particles. Schematic representation of the three particulate formulations: (1) Adilipoline-adjuvanted chitosan particles entrapping inactivated PRRSV-1 Olot/91 (Virus-P), (2) Adilipoline-adjuvanted chitosan particles entrapping PRRSV-1 M and NSP5 peptides and coated in PRRSV-1 EM4 protein (Peptide-P) and (3) empty chitosan particles (Empty-P) (**A**). Assessment of particle morphology and size by SEM (**B**). Representative SEM micrographs of each of the particulate formulations (Batch 2 – booster preparation) with scale bars indicating that the diameter of the majority of particles was in the low micrometre range. The surface charge of the three particle formulations prepared for the prime (Batch 1- filled bars) and booster (Batch 2 – open bars) immunisations were assessed by zeta potential measurements (**C**). Results presented are the mean of triplicate measurements of the same sample and error bars represent SEM. The means were compared using a one-way ANOVA followed by a Dunnett's multiple comparison test; ****$p < 0.0001$ and ***$p < 0.001$.

Figure 2. Assessment of the cytokine response profiles from porcine DCs and monocytes pulsed with vaccine particle formulations. Cultures of enriched blood DCs and monocytes were cultured for 18 hours in the presence of titrations of particle formulations (particle: cell ratios from 10:1 - 0.3:1). Both Peptide- and Virus-P were tested with ('washed') and without ('unwashed') washing to remove unbound Adilipoline. No stimulation and soluble Adilipoline were included as negative and positive controls, respectively. After incubation, the levels of IL-8, IL-10, IL-12 and type I IFN were quantified in cell-free culture supernatants by cytokine ELISA or bioassay. The results presented are the mean unstimulated

corrected values from triplicate cultures and error bars represent SEM. Log transformed cytokine responses of adjuvanted particles were compared against the Empty-P using a 2-way ANOVA with Dunnett's multiple comparison test; statistically significant differences are indicated as: 'a' - unwashed Virus-P; 'b' - washed Virus-P; 'c' - unwashed Peptide-P; and 'd' - washed Peptide-P.

Figure 3. Assessment of PRRSV and EM4 specific serum antibody responses following vaccination and challenge. Longitudinal serum samples were analysed for PRRSV specific antibody using a commercial PRRSV N-protein based ELISA (**A**) and assessed for EM4-specificity using an in-house ELISA (**B**). Data presented are the mean ELISA OD values for each group (n=6) and error bars represent SEM. Responses of each group were compared against using a 2-way ANOVA with Dunnett's multiple comparison test; statistically significant differences between the responses of Virus-P immunised pigs and Empty-P immunised pigs are indicated as 'a' whereas significant differences between Peptide-P and Empty-P immunised pigs are indicated as 'b'. The timings of booster immunisation and PRRSV-1 challenge infection are indicated.

Figure 4. Assessment of PRRSV, M and NSP5 specific T cell cytokine responses post-vaccination and challenge. Longitudinally collected PBMC were stimulated *in vitro* with peptides representing the PRRSV M and NSP5 proteins or with live PRRSV Olot/91. IFN-γ and TNF-α co-expression by CD4 (open symbols) and CD8 (closed symbols) T cells was assessed using flow cytometry (**A**). Data represent the mean background corrected response for each group (n=6) ± SEM. Responses of each group were compared against using a 2-way ANOVA with Dunnett's multiple comparison test; statistically significant differences between the responses of Virus-P immunised pigs and Empty-P immunised pigs are indicated as 'a' whereas significant differences between Peptide-P and Empty-P immunised pigs are indicated as 'b'. The timings of booster immunisation and PRRSV-1 challenge infection are

indicated as ‘B’ and ‘C’, respectively. Antigen-specific cytokine responses of individual animals were also assessed by comparing responses against those measured pre-vaccination and the proportion of animals mounting significant T cell responses post-vaccination and post-challenge are presented (**B**).

Figure 5. Assessment of viraemia post-challenge by intranasal inoculation with PRRSV-1. Serum samples were longitudinally collected post-challenge with PRRSV-1 field isolate 215-06 and PRRSV RNA assessed by quantitative RT-PCR. Data are presented as the mean PRRSV ORF7 copy numbers for each group (n=6) ± SEM. Responses of each group were compared using a 2-way ANOVA with Dunnett’s multiple comparison test; statistically significant differences between Peptide-P and Empty-P immunised pigs are indicated as ‘b’.

Figure 6. Assessment of antigen-specific lung T cell responses following intranasal challenge with PRRSV-1. Twenty one days after challenge PRRSV-1 strain 215-06, T cells in BALF were phenotyped and enumerated by volumetric flow cytometry (A). Isolated cells were stimulated *in vitro* with PRRSV (virus) or a pool of M and NSP5 peptides (peptide). IFN-γ and TNF-α expression by CD4 and CD8 T cells was assessed by flow cytometry (B). Data represent the mean responses of animals in each group from which lavage fluid could be collected without contaminating blood cells (Virus-P n=5; Peptide-P n=1; Empty-P n=6), with bars representing SEM. Differences between CD4 and CD8 responses from all animals were compared using a one-way ANOVA followed by a Dunnett’s multiple comparison test; ***$p < 0.001$ and **$p < 0.01$.

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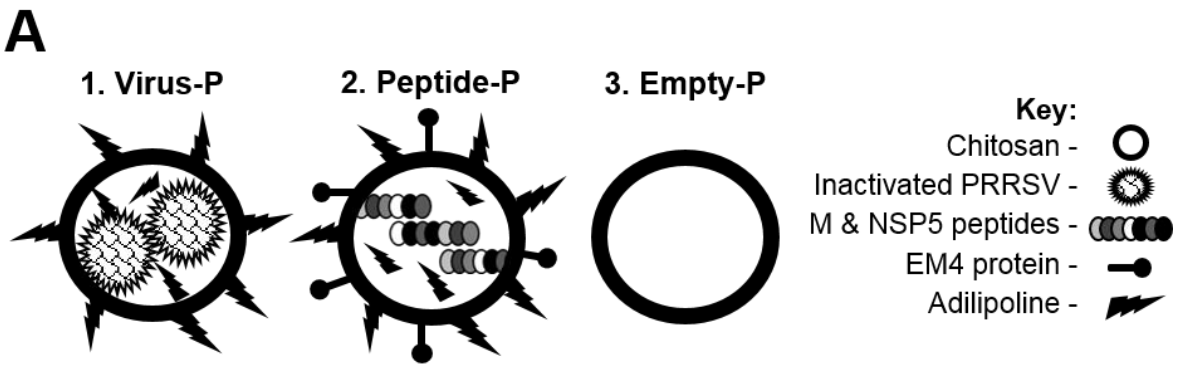
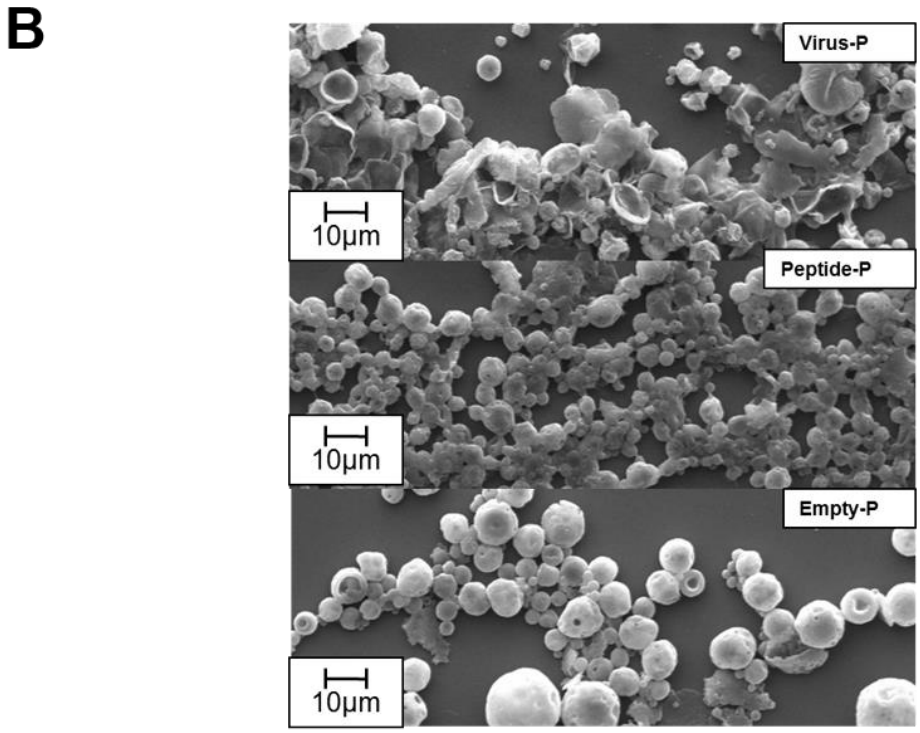
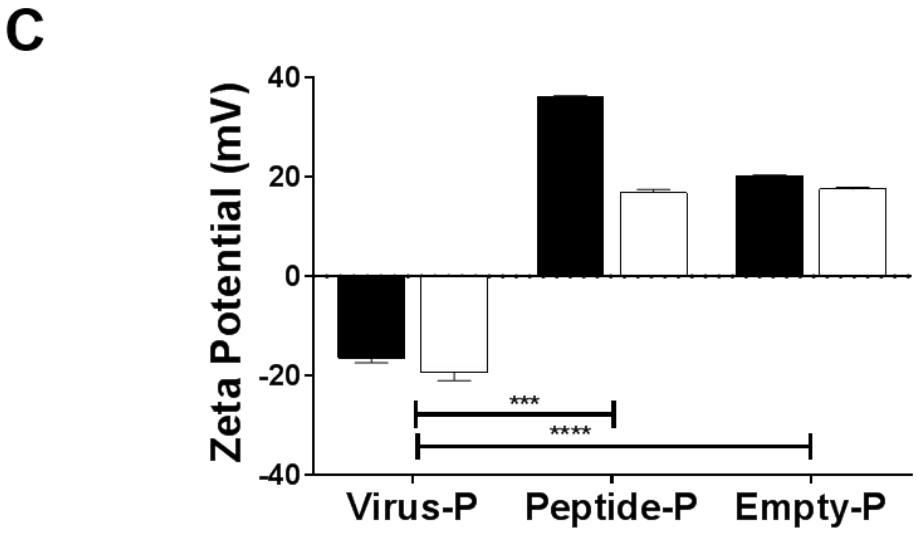
619 FIGURE 1







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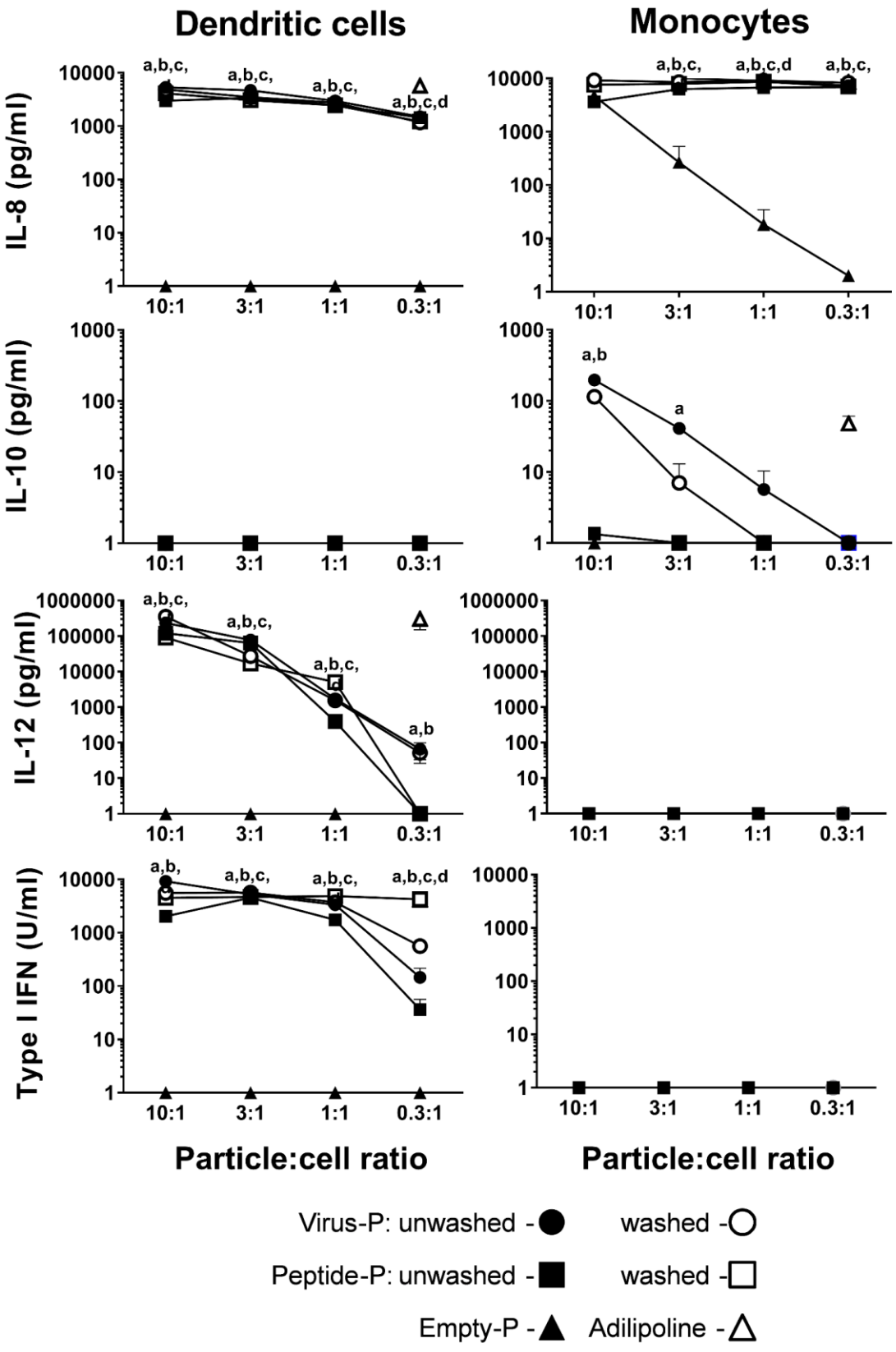
621 **FIGURE 2**



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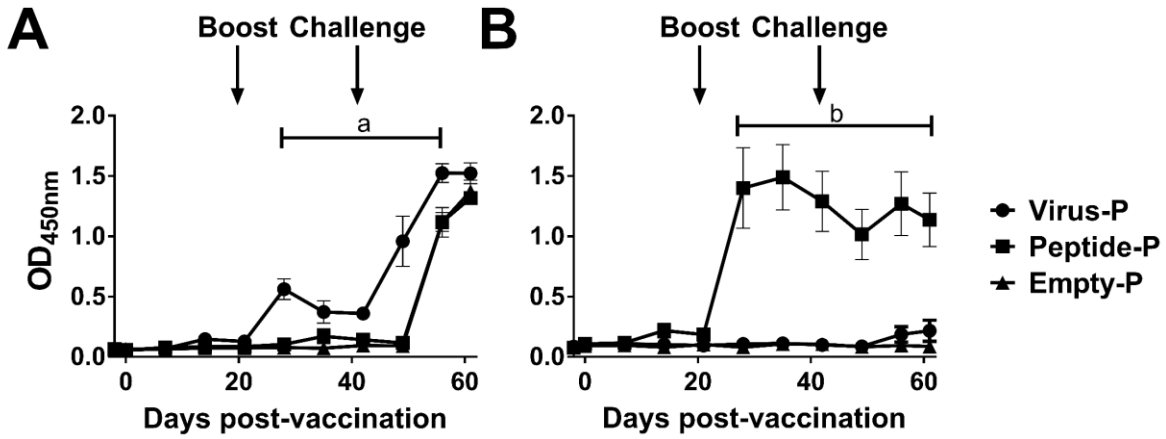
623 **FIGURE 3**



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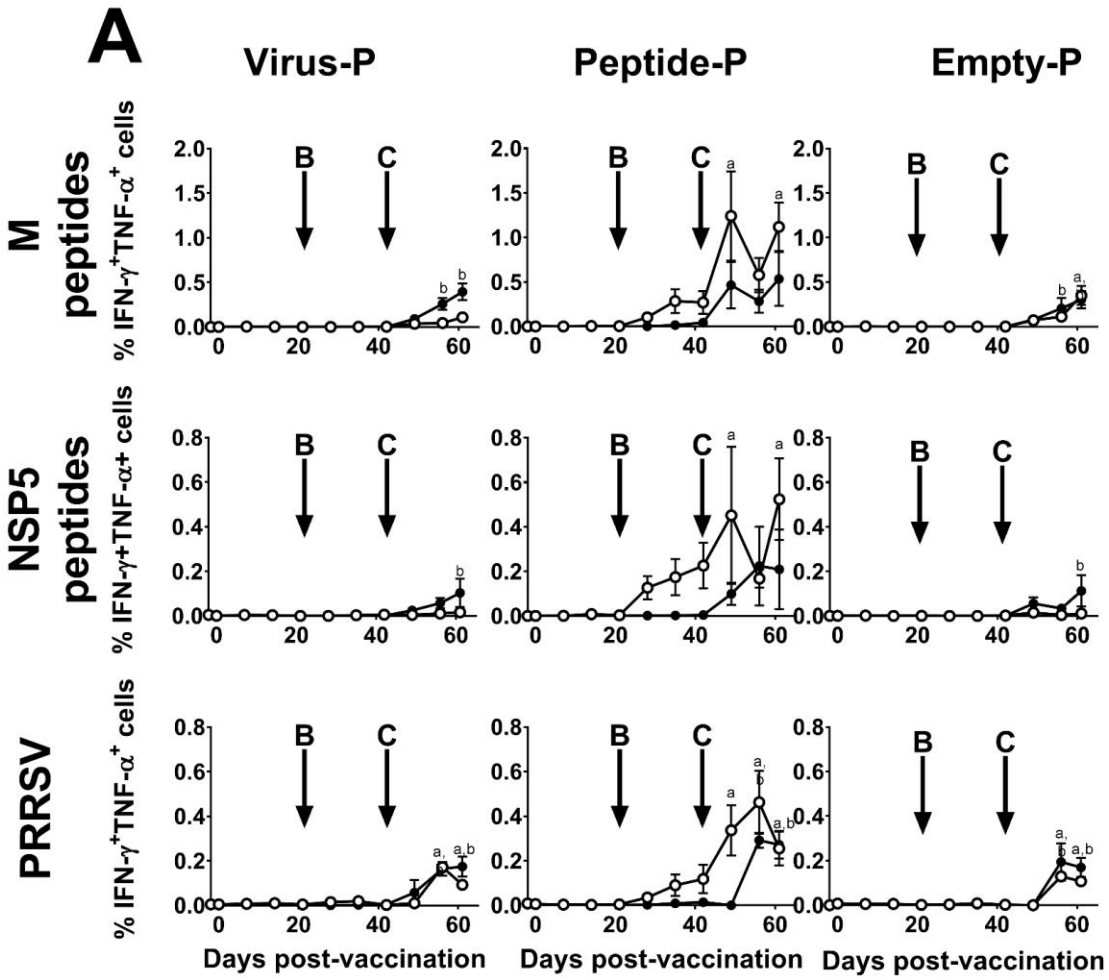
626 **FIGURE 4**



B

Group (n=6)	Proportion of animals displaying significant antigen -specific T cell responses											
	Post-vaccination						Post-challenge					
	M		NSP5		PRRSV		M		NSP5		PRRSV	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Virus-P	0	0	0	0	1	0	4	6	1	2	5	6
Peptide-P	3	0	3	0	1	0	6	4	4	3	5	5
Empty-P	0	0	0	0	0	0	5	5	0	4	6	6

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FIGURE 5

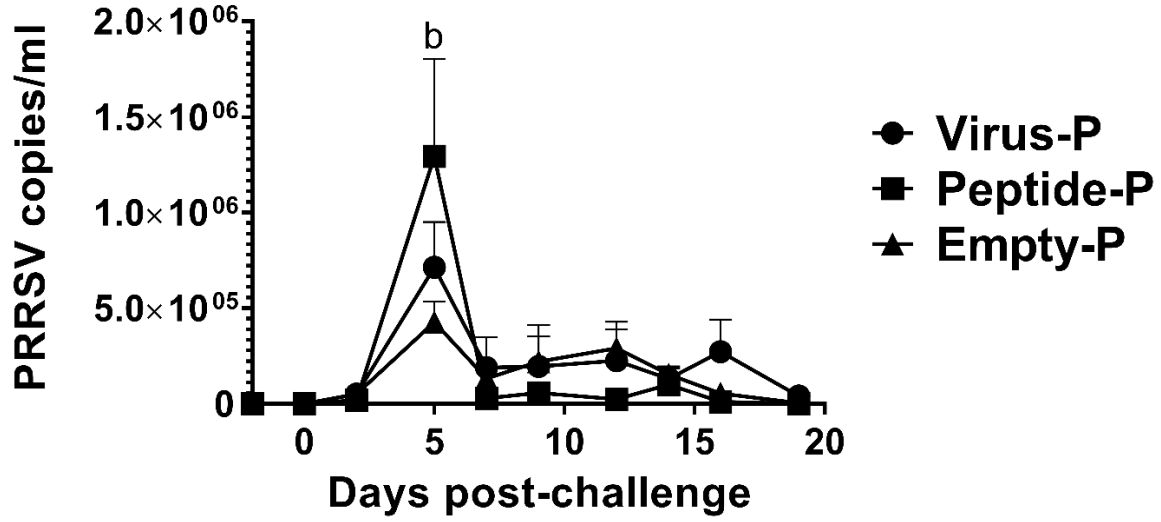
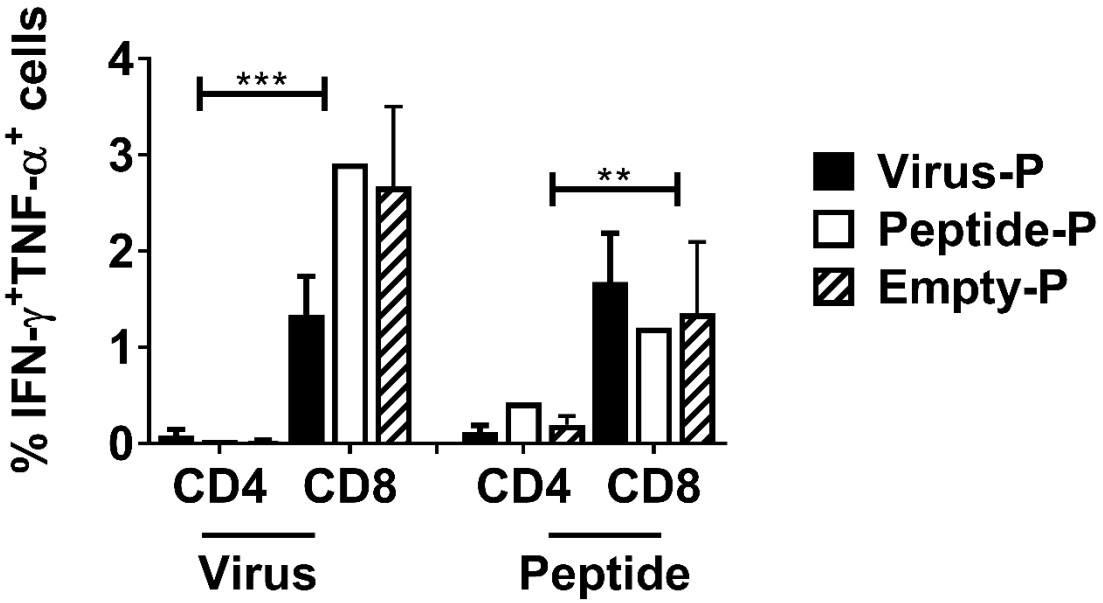


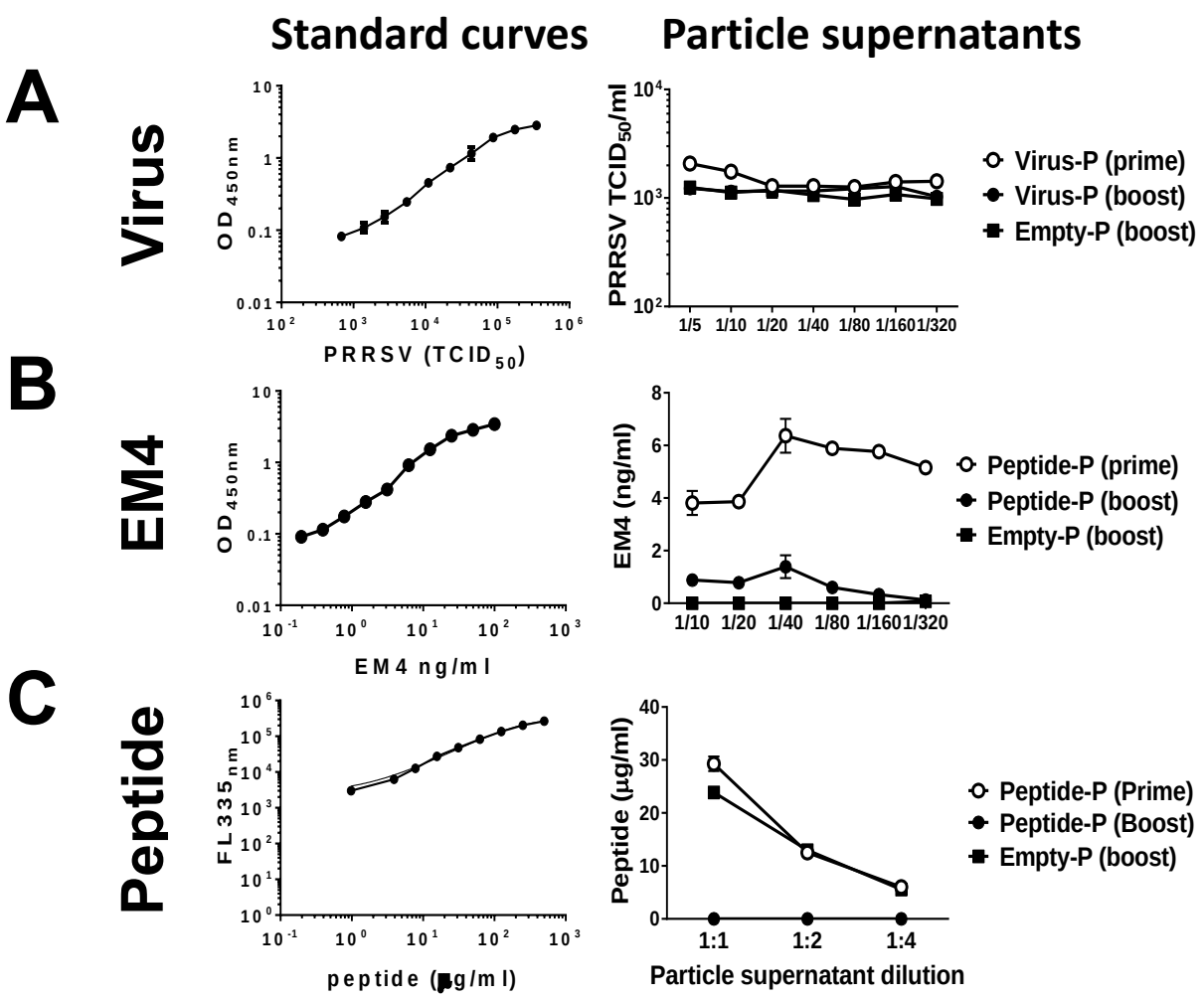
FIGURE 6



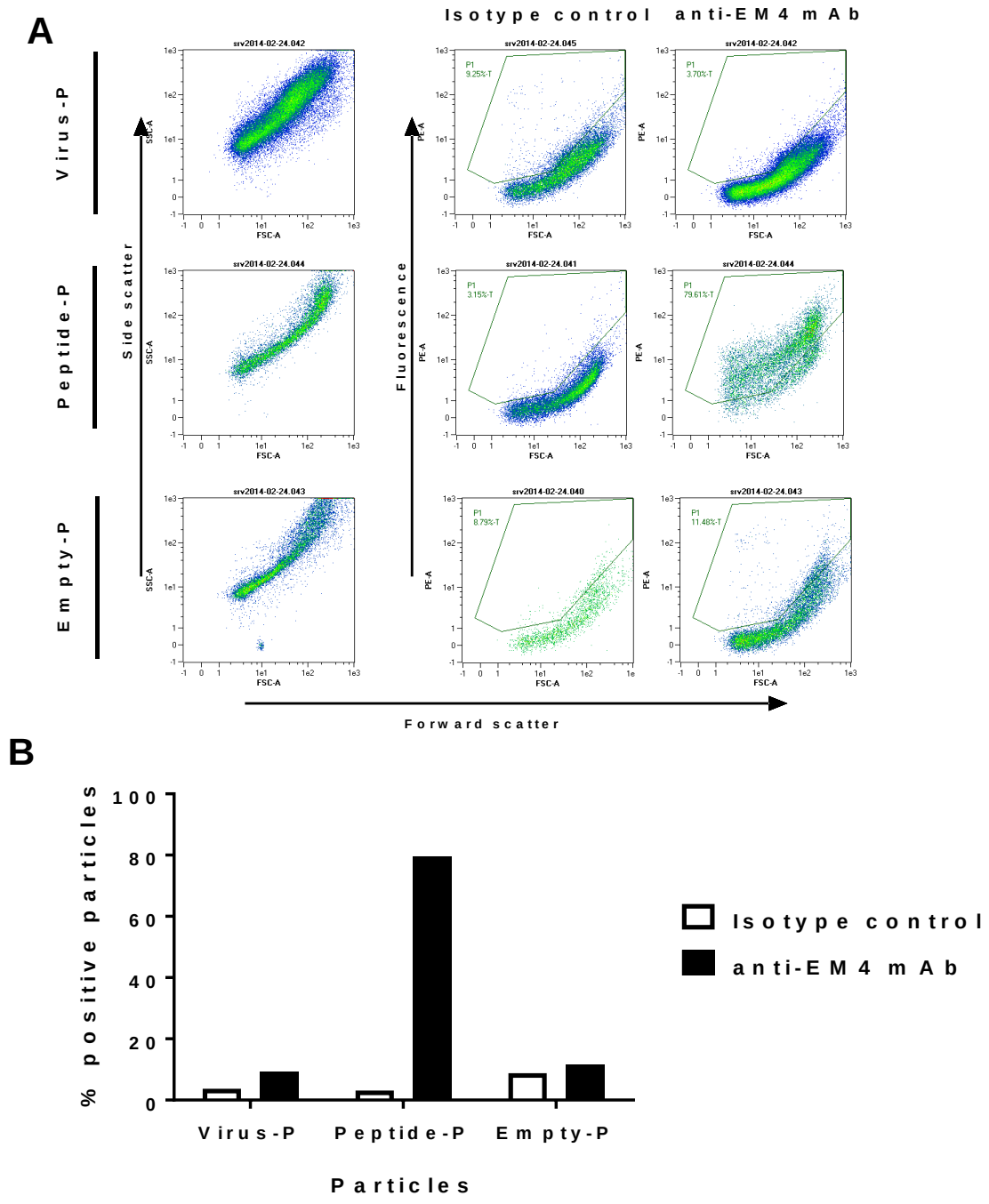
Supplementary Materials

Evaluation of hydrophobic chitosan-based particulate formulations of porcine reproductive and respiratory syndrome virus vaccine candidate T cell antigens

Helen Mokhtar, Lucia Biffar, Satyanarayana Somavarapu, Jean-Pierre Frossard, Sarah McGowan, Miriam Pedrera, Rebecca Strong, Jane C. Edwards, Margarita Garcia-Durán, Maria Jose Rodriguez, Graham R. Stewart, Falko Steinbach, Simon P. Graham



Supplementary Figure S1. Assessment of inactivated PRRSV-1, EM4 and peptide incorporation into particle formulations. The presence of inactivated PRRSV-1 (Virus) (**A**) and EM4 protein (**B**) in particle-free supernatants were assessed by antigen-capture ELISAs. The presence of M and NSP5 peptides in particle-free supernatants was assessed by fluorometric OPA assay (**C**)**.** Representative standard curves for each assay (left column) are presented as are the mean triplicate values of titrations of particle supernatants from preparations formulated for prime and boost immunisations and error bars represent SEM (right column).



Supplementary Figure S2. Assessment of EM4 coating of chitosan particle formulations by flow cytometry. The absorption of EM4 protein onto the surface of chitosan particles was assessed by staining particles with a specific mAb and visualisation by staining with a fluorochrome labelled secondary antibody. Representative dot plots are shown of the forward and side scatter properties of each of the three particle formulations and their staining following incubation with isotype control or EM4 specific mAbs (**A**). The percentage of particles falling into the positive staining gate are graphically presented in panel **B**.

Supplementary Materials and Methods

Assessment of the antigen loading efficiency

Aliquots of each particle formulation were centrifuged at 14000rpm for 5 min and supernatants were harvested and stored at -80°C until analysed. ELISA and biochemical assay analyses of particle-free supernatants were conducted to determine the efficiency of virus or peptide encapsulation and coating with EM4 protein. Absorption of EM4 to particle surfaces was additionally assessed by flow cytometric staining and analyses of particles.

EM4 protein in particle-free supernatants was assessed by ELISA. ELISA plates (MAXISorb, Nunc, Fisher Scientific, Loughborough, UK) were coated (100µl/well) overnight at 4°C with titrations of particle-free supernatants from Peptide- and Empty-P formulations in carbonate-bicarbonate buffer. Recombinant EM4 protein was included as a standard. Plates were washed, blocked PBS, 0.05% Tween, 5% BSA, and incubated with 1 µg/ml anti-EM4 mAb (Clone EM11E10, Ingenasa, Madrid Spain). After 1 h incubation at room temperature, plates were washed and incubated with rabbit-anti-mouse IgG-HRP antibody (Dako, Ely, UK) 1h at room temperature. Subsequently, plates were washed, incubated with TMB substrate (Sigma-Aldrich) for a maximum of 10 min in the dark before the reaction was stopped with 0.5M H_2SO_4 and absorbance at 450nm read on a Victor$_4$ microplate reader (Perkin Elmer, Seer Green, UK). ELISA quantification of inactivated PRRSV-1 in particle-free supernatants was performed as described above with the following modifications: plates were coated with particle-free supernatants from Virus- and Empty-P formulations; inactivated PRRSV Olot/91 was used as a standard; antigen was detected using anti-PRRSV N protein mAb clone 1AC7 at 10µg/ml (Ingenasa). To quantify peptides in particle-free supernatants the biochemical fluorometric o-phthalaldehyde (OPA) assay was performed according to manufacturer's instructions (Fluoraldehyde (OPA) Reagent Solution, Thermo Scientific, UK) with minor modifications: particle-wash supernatants from Peptide- and Empty-P formulations were serially diluted two-fold in water; a pool of M and NSP5 peptides was used as a standard; 20µl of samples or standards were mixed with 200 µl of Fluoraldehyde Reagent Solution in black 96 well microplates (OptiPlate; PerkinElmer) and fluorescence read immediately on a Victor$_4$

725 microplate reader using excitation and emission wavelengths of 340 and 455nm,
726 respectively.
727 To assess absorption of EM4 protein to particles by flow cytometry, particles were
728 washed twice in PBS and stained 1µg/ml anti-EM4 mAb (E10C7) or isotype control
729 IgG2b antibody (AbD Serotec, Oxford, UK) for 30 min at 4oC. After two washes in
730 particles were stained with anti-mouse IgG2-PE conjugated secondary antibody (BD
731 Biosciences) and incubated as before. After a final two washes, particles were
732 analysed on a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bisley, UK).
733 Particles were gated by forward and side scatter measurements with logarithmic
734 scaling. EM4 staining was assessed by setting a gate based on isotype control mAb
735 staining.
736