Supplementary Material

An attenuated herpesvirus vectored vaccine candidate induces T cell responses against highly conserved porcine reproductive and respiratory syndrome virus M and NSP5 proteins that are unable to control infection

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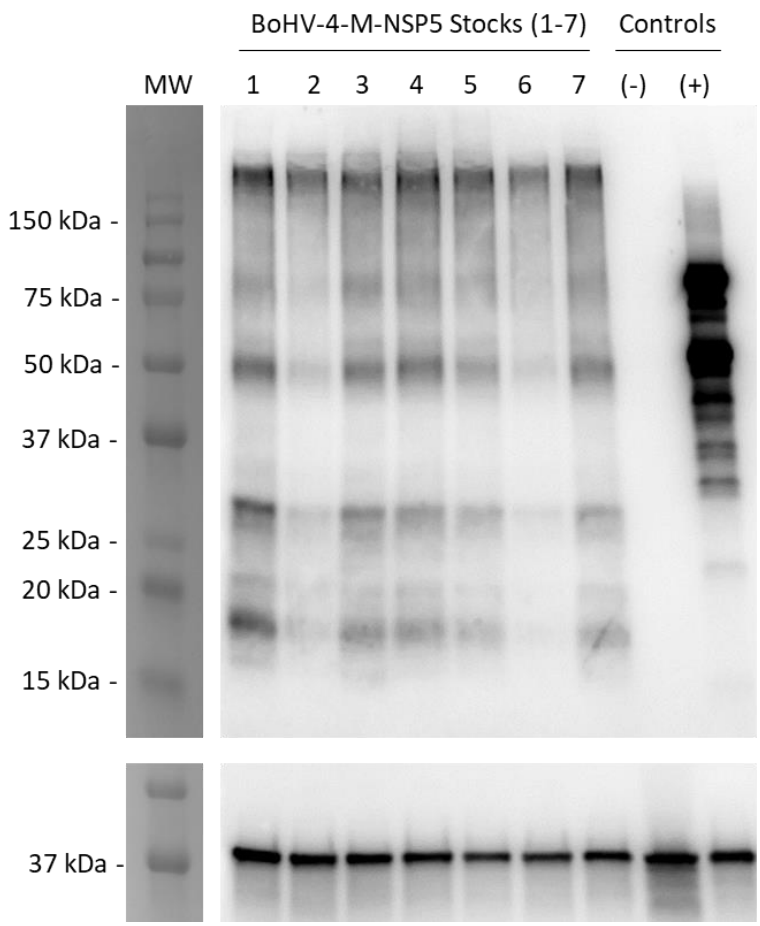


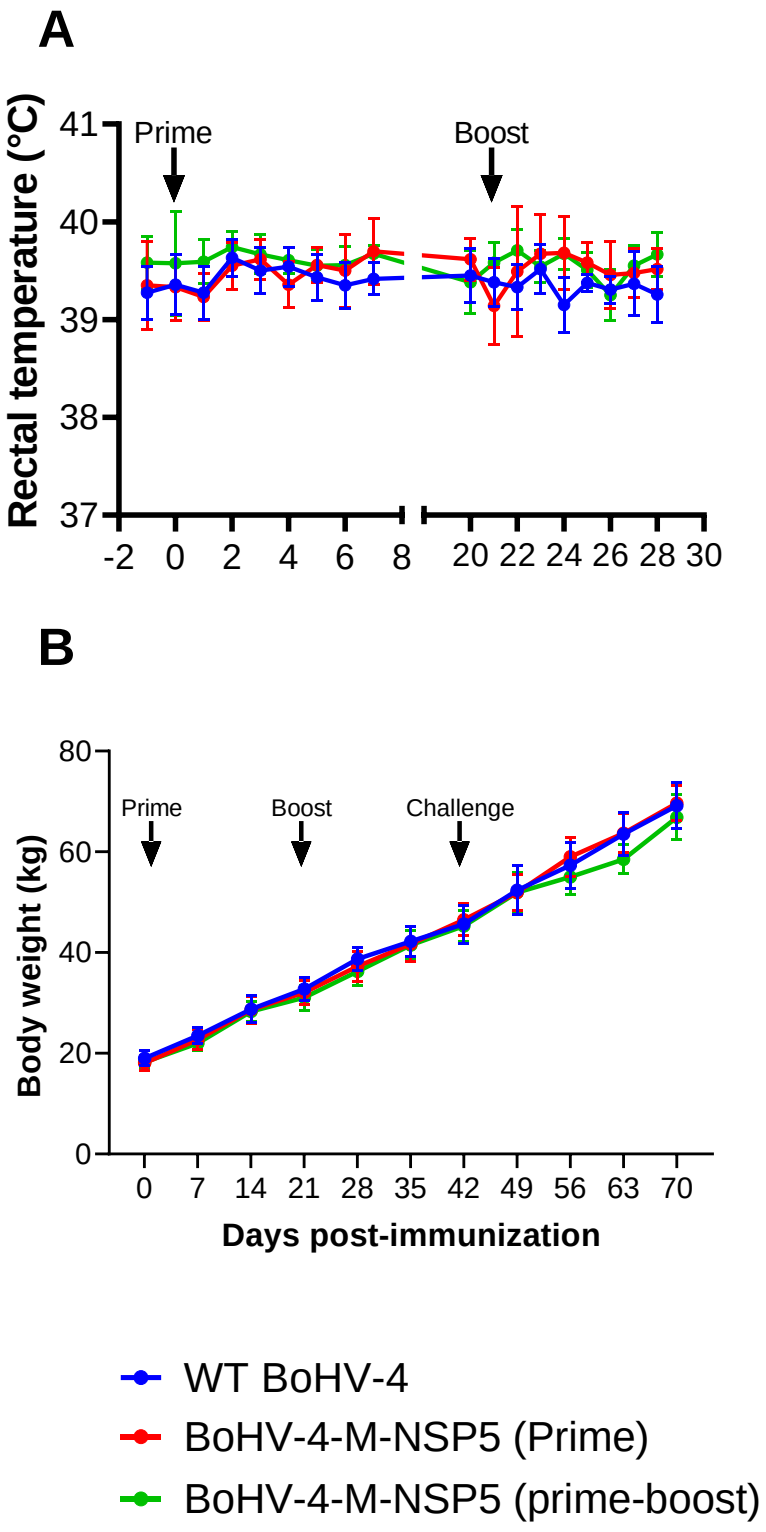
Supplementary Figure 1. Western immunoblot showing expression of M-NSP5 fusion protein by BoHV-4-M-NSP5. MDBK cells were infected with concentrated BoHV-4-M-NSP5 virus stocks (stocks 1-7). The figure shows western immunoblot analysis of cell lysates with multiple V5-reactive bands, in addition to the expected band size of 50 kDa, likely due to protein multimerization and proteolysis. Lysates were not heated to avoid excessive M-NSP5 aggregation. M-NSP5 was detected using an antibody directed against the C-terminal V5 epitope tag. A BoHV-4 WT (-) was used as a negative control and a BoHV-4 recombinant expressing a V5-tagged bovine tuberculosis antigen (+) was used as a positive control. An antibody directed against cellular p38 protein was used as a loading control.

Supplementary Table 1 – For study 1, the clinical score system comprised the below parameters measured daily for a week after each vaccination and for a maximum of 14 days post-challenge.

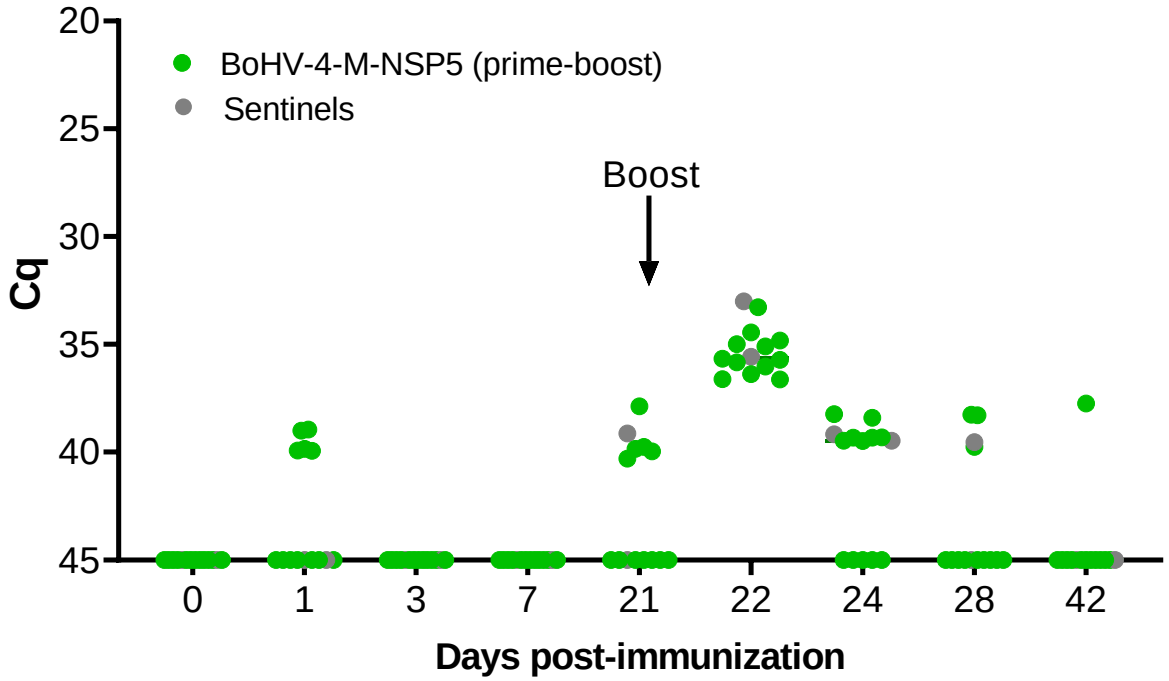
Parameter		*Criteria*		**Score**
1	**Skin changes**	1a. Redness/ purpling of skin of ears	Absent/Present	***0/1***
		1b.Reddening/purpling of skin elsewhere on body	Absent/Present	***0/1***
		1c. Ear necrosis	Absent/Present	***0/2***
2	**Behaviour**	2a. Alertness/Movement	Get up quickly look alert to what is happening/eyes open	***0***
			Lethargic	***1***
			Get up slowly not as alert, eyes closed	***2***
			No attempt to get up even when touched, eyes closed	***6***
		2b. Interaction with others	Normal	***0***
			Reduced	***1***
			None	***2***
		2c. Investigating toys	Keen interest in toys/rope	***0***
			Limited interest in toys/rope	***1***
			No interest	***2***
3	**Eyes/conjunctiva**	3a. Ocular discharge	Absent/Present	***0/1***
		3b. Swelling of eyelids	Absent/Present	***0/1***
4	**Nasal discharge**		Absent	***0***
			Present and clear	***1***
			Present and discoloured	***2***
5	**Respiratory changes**	Frequency 10-15/min, barely visible chest movement		***0***
		Frequency >20/min distinct chest and abdominal movement		***2***
		Frequency >30/min, breathing through open mouth		***6***
6	**Coughing**	None		***0***
		Mild (few incidences as 1-5)		***1***
		Severe (more frequent incidences >5)		***2***
7	**Defecation**	Soft faeces, normal amount		***0***
		Reduced amount of faeces, dry		***1***
		No faeces, mucus in rectum or diarrhoea		***2***
8	**Appetite**	Hungry, trough empty, clean		***0***
		Reduced eating		***1***
		Only picking at food		***4***
		Does not eat when fed		***6***
9	**Body Condition**	Good		***0***
		Fair		***1***
		Poor (ribs and/or backbone showing)		***2***
10	**Temperature**			
11	**Other**	Note any additional observations:		

Supplementary Table 2 – For study 2, the clinical score system comprised the below parameters measured daily for a week after each vaccination and for a maximum of 10 days post-challenge.

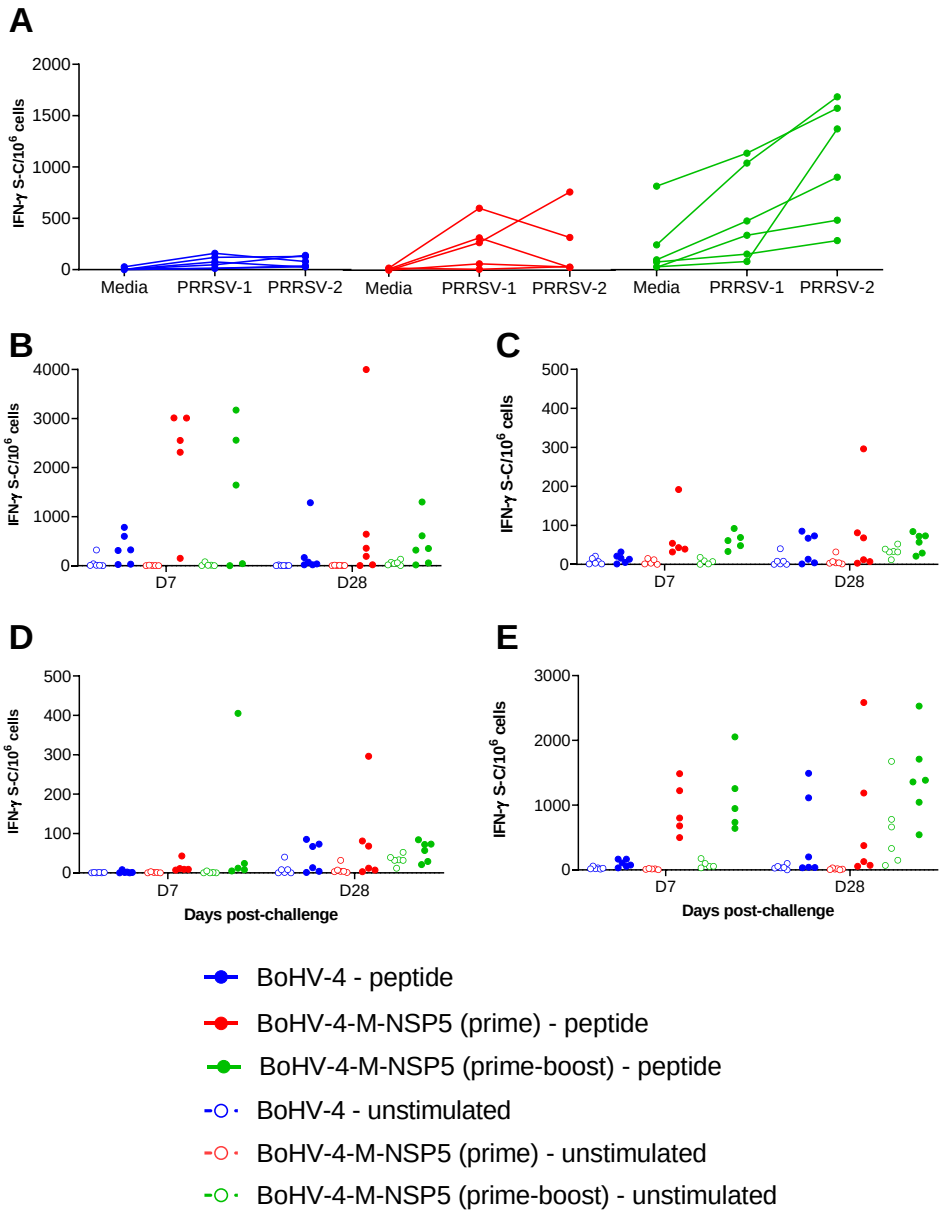
	Parameter	Criteria	Score
1	Skin changes		
1a	Reddening/purpling of skin of ears	Absent/present	0/1
1b	Reddening/purpling of skin elsewhere on body	Absent/present	0/1
1c	Ear necrosis	Absent/present	0/2
2	Behaviour		
2a	Alertness/Movement	Get up quickly look alert to what is happening/eyes open	0
		Lethargic	1
		Get up slowly not as alert, eyes closed	2
		No attempt to get up even when touched, eyes closed	6
2b	Interaction with others	Normal	0
		Reduced	1
		None	2
2c	Investigating toys	Keen interest in toys/rope	0
		Limited interest in toys/rope	1
		No interest	2
3	Eyes/conjunctiva		
3a	Ocular discharge	Absent/Present	0/1
3b	Swelling of eyelids	Absent/Present	0/1
4	Nasal discharge	Absent	0
		Present and clear	1
		Present and discoloured	2
5	Respiratory changes	Frequency 10-15/min, barely visible chest movement	0
		Frequency >20/min distinct chest and abdominal movement	2
		Frequency >30/min, breathing through open mouth	6
6	Coughing	None	0
		Mild (few incidences as 1-5)	1
		Severe (more frequent incidences >5)	2
7	Defecation	Soft faeces, normal amount	0
		Reduced amount of faeces, dry	1
		No faeces, mucus in rectum or diarrhoea	2
8	Appetite	Hungry, trough empty, clean	0
		Reduced eating	1
		Only picking at food	4
		Does not eat when fed	6
9	Body Condition	Good	0
		Fair	1
		Poor (ribs and/or backbone showing)	2
10	Other	Note any additional observations	



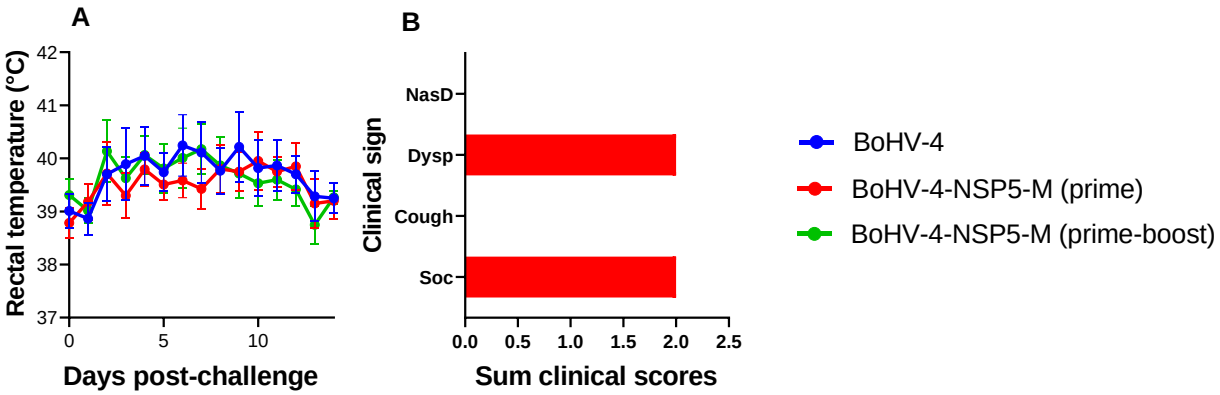
Supplementary Figure 2. Evaluation of the safety of a BoHV-4 vectored PRRSV vaccine candidate. In study 1, rectal temperatures measured daily for 7 days post-prime and -boost immunization (**A**). Animals were weighed weekly post-vaccination and -challenge **(B)**. Mean data ± SD for each group are shown.



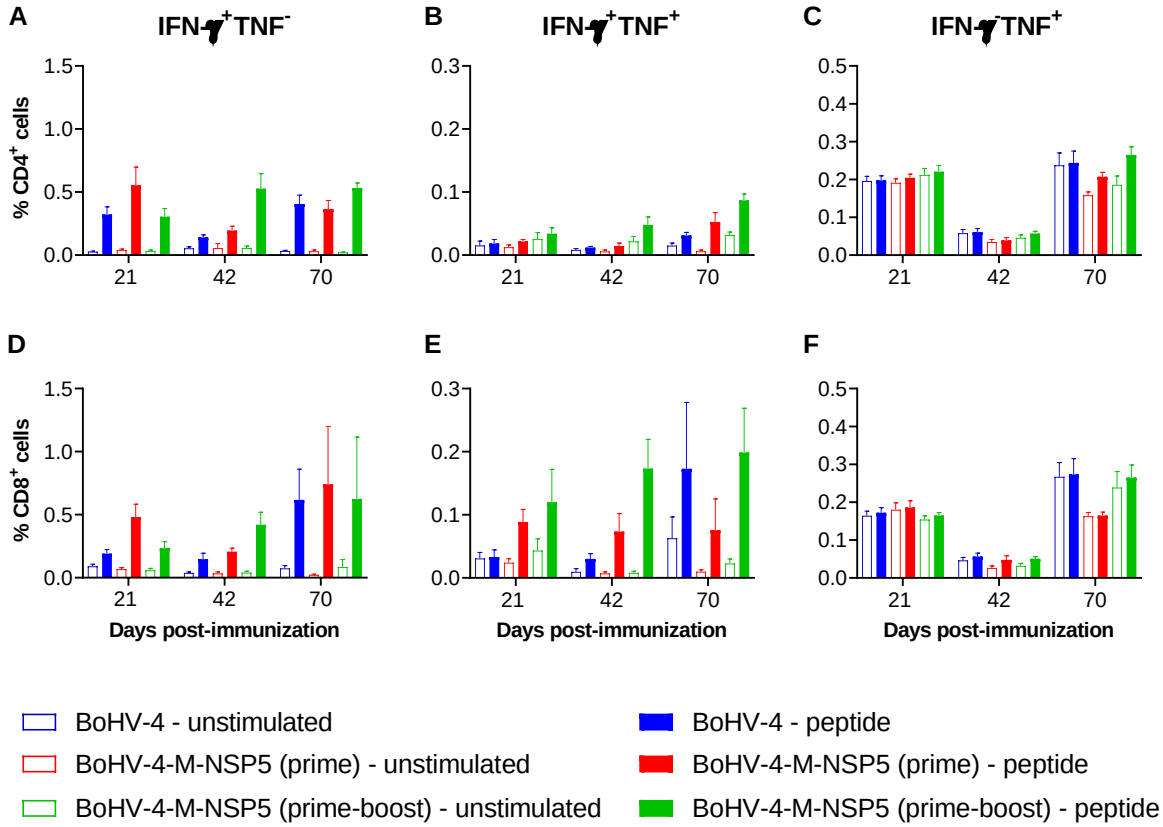
Supplementary Figure 3. Assessment of BoHV-4 shedding genome detection in nasal swabs. BoHV-4 genome in nasal swabs was inferred by qPCR analysis of pigs immunized with BoHV-4-M-NSP5 (prime-boost) from study 1. Swabs from co-housed sentinel pigs were included in the analysis. Data are presented as C_q values, datapoints represent individual pigs with the median indicated by a horizontal line.



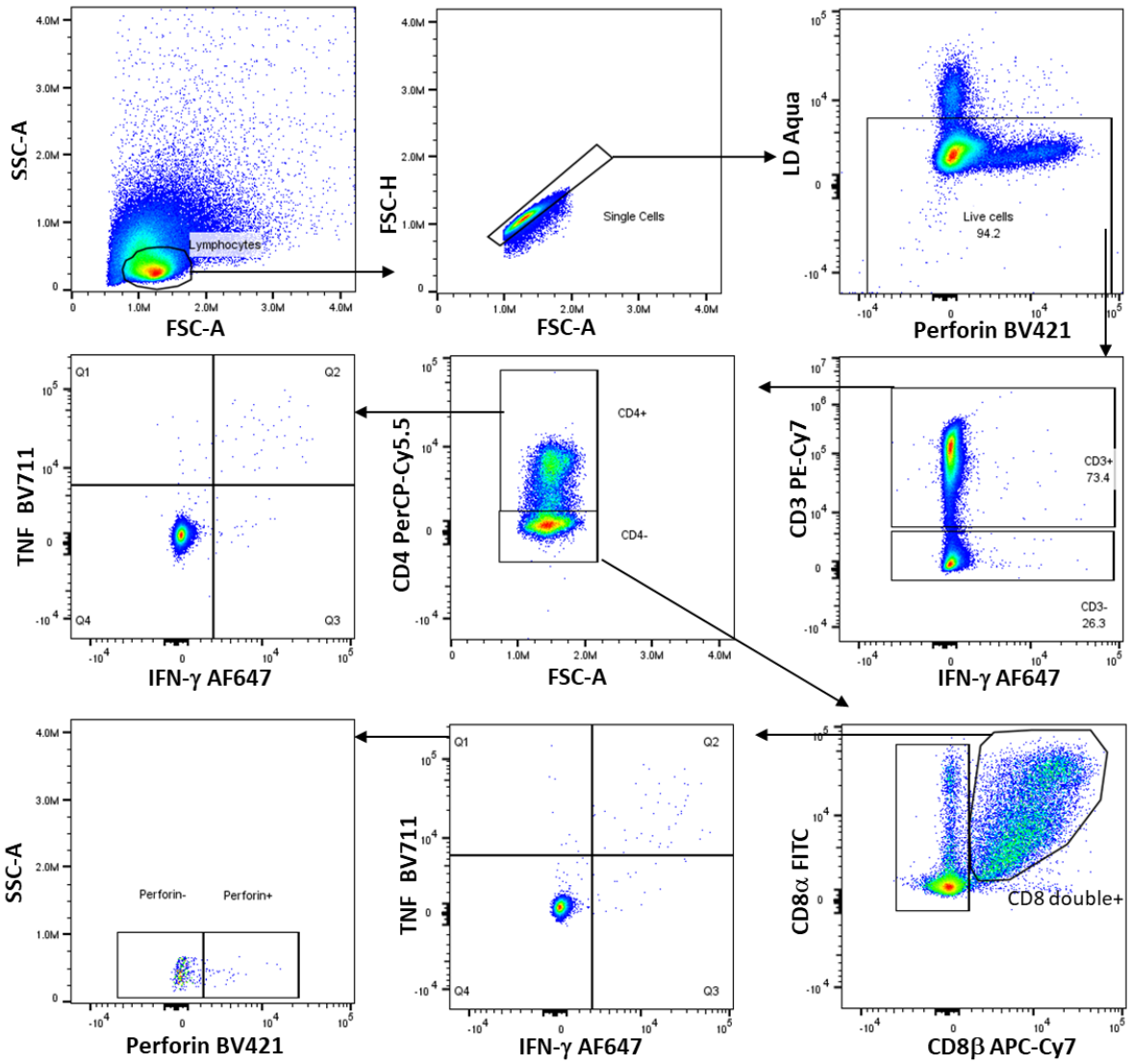
Supplementary Figure 4. Assessment of IFN-γ responses induced by immunization with a BoHV-4 vectored PRRSV vaccine candidate and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific IFN-γ responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (study 1). Control pigs were immunized twice with the WT BoHV-4 vector. PBMC responses were assessed following peptide stimulation by IFN-γ ELISpot assay. PBMC responses were assessed on D63 by stimulation with peptide pools representing M/NSP5 from PRRSV-1 and -2 (**A**). Responses were additionally assessed from cells isolated from BAL (**B**), thymus (**C**), inguinal lymph nodes (**D**) and spleen (**E**) post-challenge. Datapoints represent the unstimulated or peptide-stimulated IFN-γ spot-forming cells (S-C) per million cells for individual pigs.



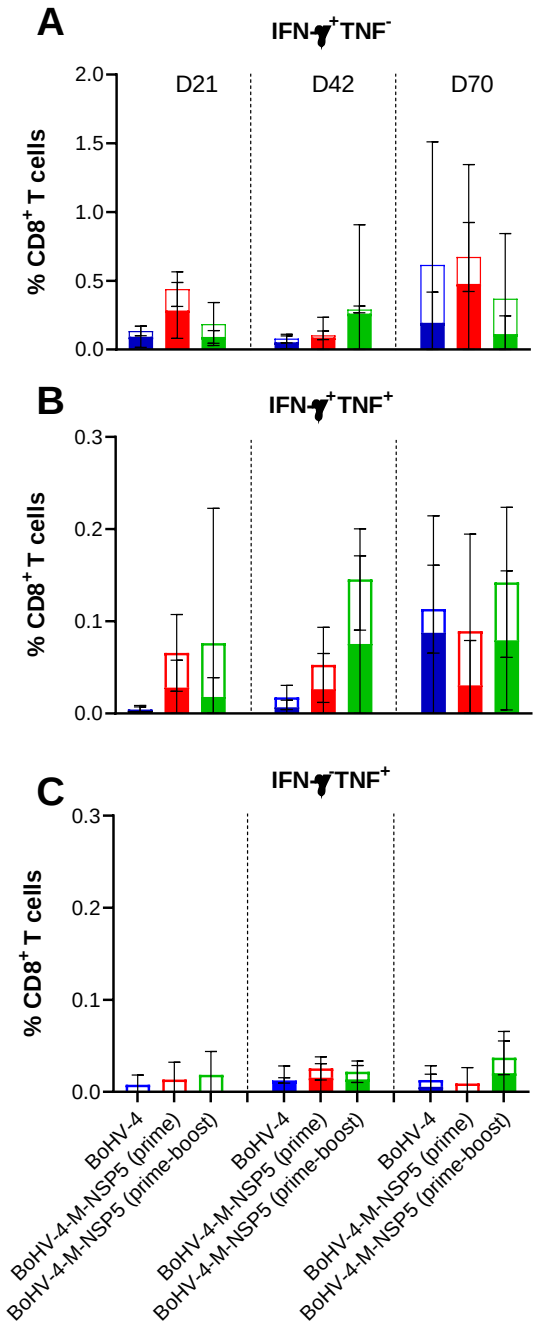
Supplementary Figure 5. Evaluation of the efficacy of a BoHV-4 vectored PRRSV vaccine candidate. The ability BoHV-4-M-NSP5 immunization to confer clinical protection was assessed following challenge infection of pigs with PRRSV-1 21301/19 (Study 1). A negative control group comprised two immunisations with the BoHV-4 vector. Mean data ± SD for rectal temperatures over the course of challenge are shown (**A**). The sum of clinical scores observed in the post-challenge period are presented for each group (**B**). Nasal discharge – NasD; dyspnoea – Dysp, and changes in social behavior – Soc.



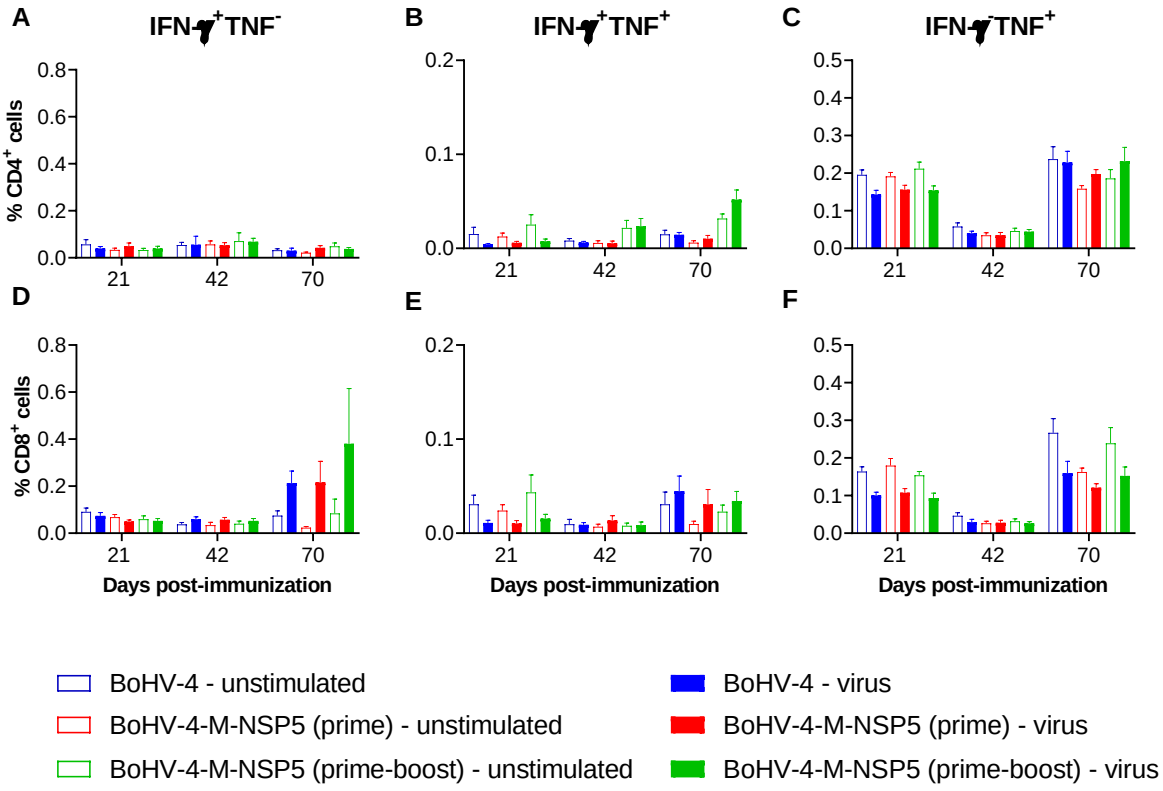
Supplementary Figure 6. Characterization of antigen-specific cytokine responses in the blood of pigs after vaccination with BoHV-4-M-NSP5 and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (Study 1). Control pigs were immunized twice with the BoHV-4 vector before the challenge. Responses of previously cryopreserved PBMCs from D21, D42 and D70 were assessed with and without peptide stimulation by flow cytometry. Expression of IFN-γ alone (**A**, **D**), IFN-γ and TNF (**B, E**), or TNF alone (**C**, **F**) by CD4 T cells (**A - C**) and CD8 T cells (**D - F**) are shown as mean data ± SD for each vaccine group.



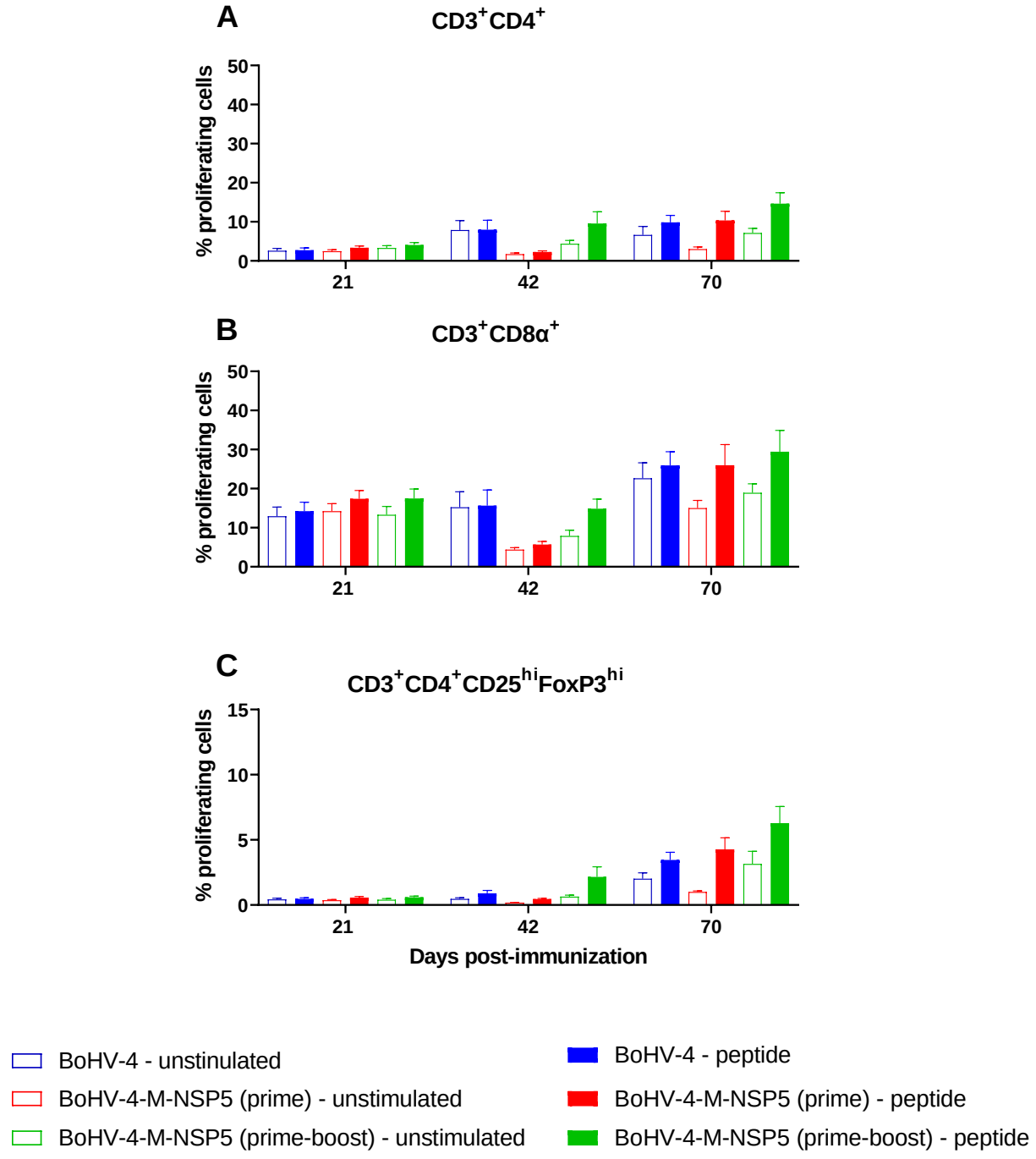
Supplementary Figure 7. Flow cytometric gating strategy for assessment of T cell cytokine responses. Lymphocytes were gated by their characteristic light scattering and then further gated to exclude dead cells. Live lymphocytes were separated into $CD3^+$ T-cells and $CD3^-$ non-T-cells before $CD3^+$ cells were sub-gated for $CD4^+$ T cells, $CD8\alpha\beta^+$ T cells, non-conventional T cells defined as $CD3^+$ $CD8\alpha^{low/+}$ $CD8\beta^-$ $CD4^-$ T-cells. Single and double IFN-γ^+ and TNF^+ cells were gated within the various $CD3^+$ populations. Finally, the perforin expression by cytokine expressing CD8 T cells was assessed.



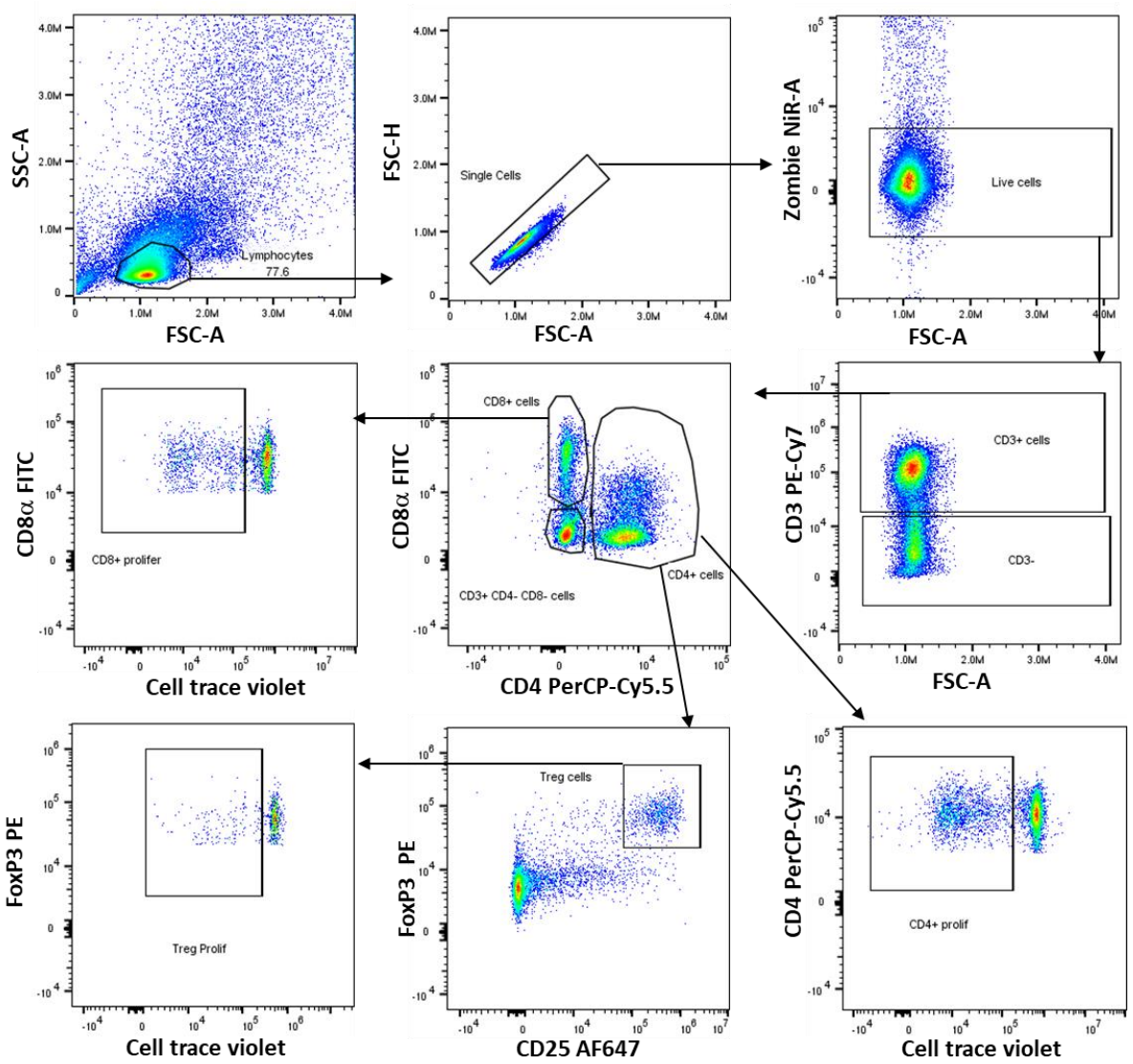
Supplementary Figure 8. Assessment of perforin expression by CD8 T cells after vaccination with BoHV-4-M-NSP5 and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (study 1). Control pigs were immunized twice with the BoHV-4 vector before PRRSV challenge. Responses of previously cryopreserved PBMCs from D21, D42 and D70 were assessed following peptide stimulation by flow cytometry. Perforin expression (perforin$^+$ - empty bars; perforin$^-$ - solid bars) by CD8 T cells expressing IFN-γ alone (**A**), IFN-γ and TNF (**B**), or TNF alone (**C**) are shown as mean unstimulated condition corrected data ± SEM for each vaccine group.



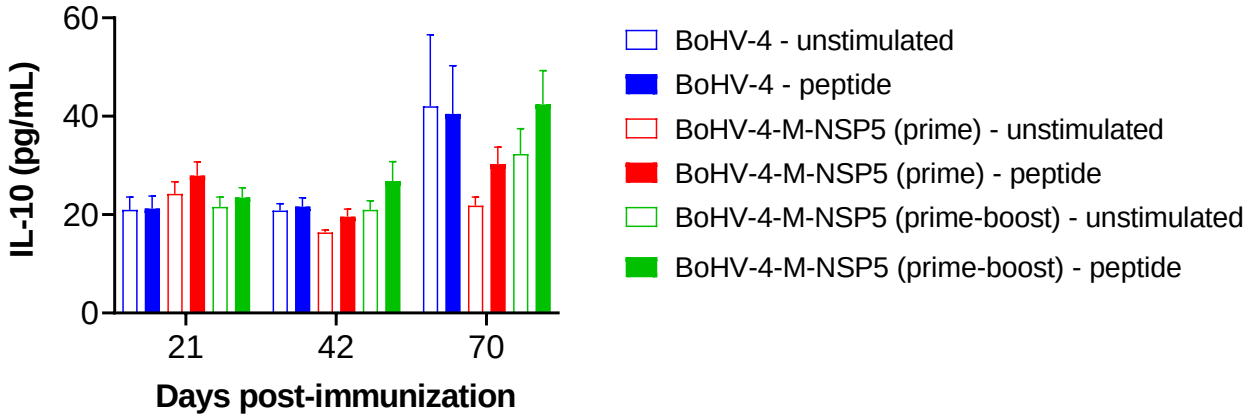
Supplementary Figure 9. Characterization of antigen-specific cytokine responses in the blood of pigs after vaccination with BoHV-4-M-NSP5 and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (Study 1). Control pigs were immunized twice with the BoHV-4 vector before PRRSV challenge. Responses of previously cryopreserved PBMCs from D21, D42 and D70 were assessed with and without PRRSV-1 21301/19 (virus) stimulation by flow cytometry. Expression of IFN-γ alone (**A**, **D**), IFN-γ and TNF (**B, E**), or TNF alone (**C**, **F**) by CD4 T cells (**A - C**) and CD8 T cells (**D - F**) are shown as mean data ± SD for each vaccine group.



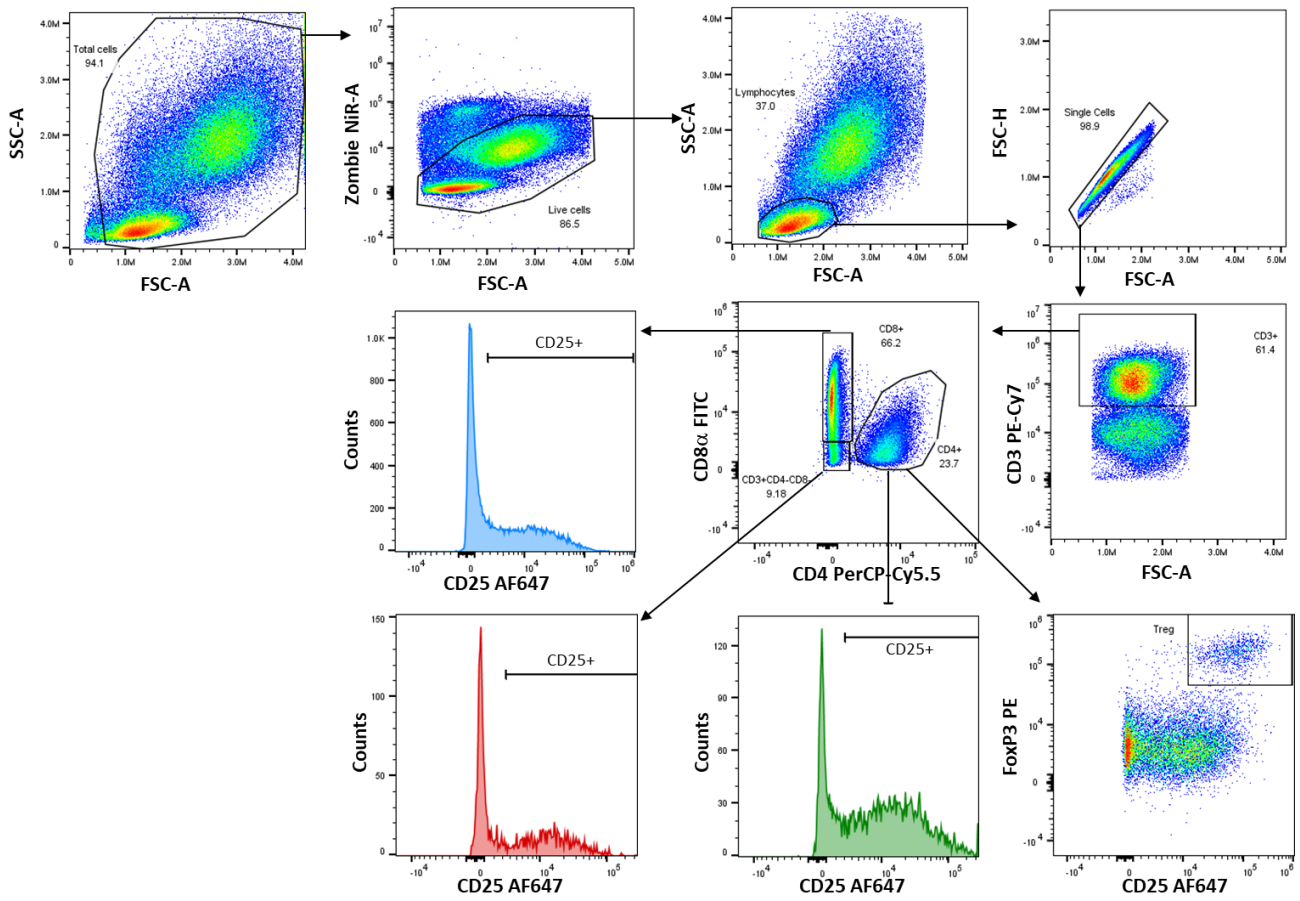
Supplementary Figure 10. Characterization of antigen-specific proliferative responses in the blood of pigs after vaccination with BoHV-4-M-NSP5 and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific proliferative responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (study 1). Control pigs were immunized twice with the BoHV-4 vector before PRRSV challenge. Responses of previously cryopreserved PBMCs from D21, D42 and D70 were assessed with and without peptide stimulation by flow cytometry. The mean % proliferation of CD4 T cells (**A**), CD8 T cells (**B**) and Tregs (**C**) are shown ± SD for each vaccine group.



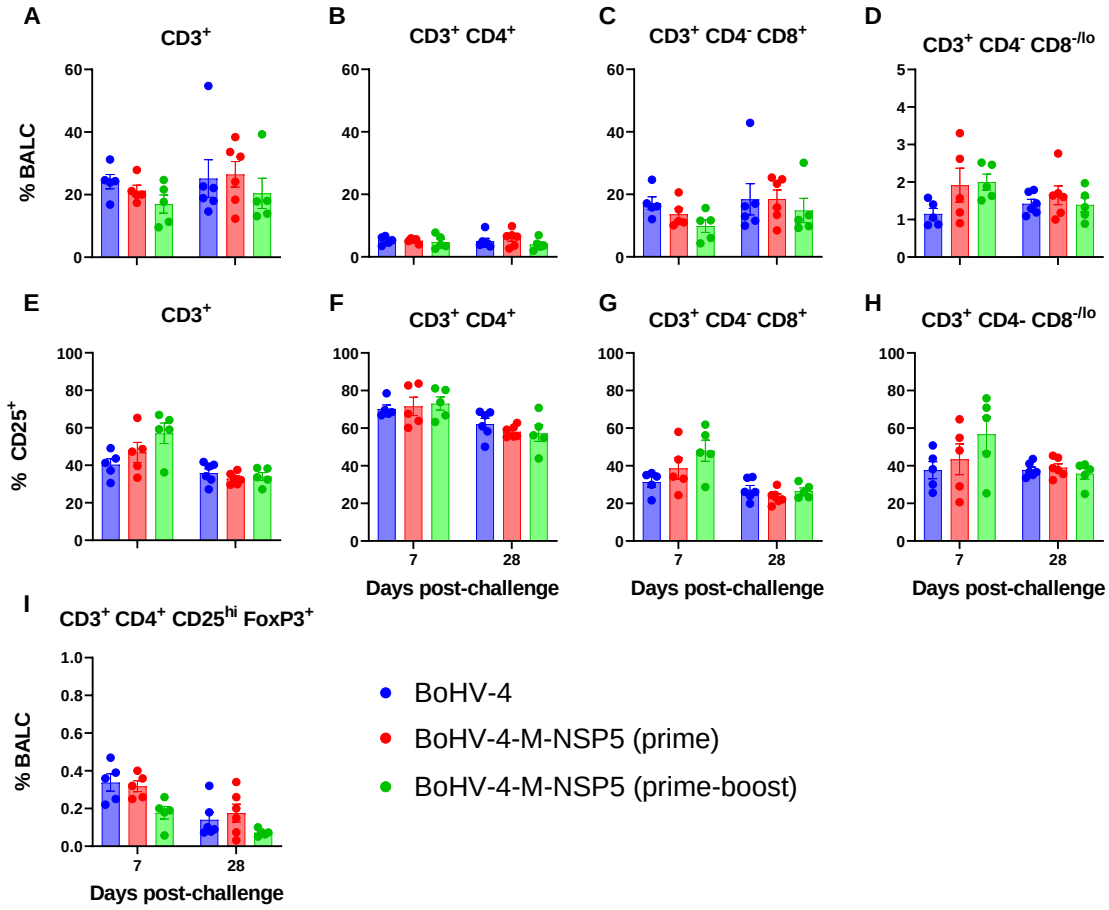
Supplementary Figure 11. Flow cytometric gating strategy for assessment of T cell proliferative responses. Lymphocytes were gated by their characteristic light scattering and then further gated to exclude dead cells. Live lymphocytes were separated into $CD3^+$ T-cells and $CD3^-$ non-T-cells before $CD3^+$ cells were sub-gated for $CD4^+$ T cells, $CD8\alpha^+$ T cells, and Tregs defined at $CD3^+$ $CD4^+$ $CD25^{high}$ $FoxP3^+$. The % of proliferating cells was assessed as those displaying reduced CellTrace™ Violet labelling. Representative dot plots from peptide stimulated PBMC are shown.



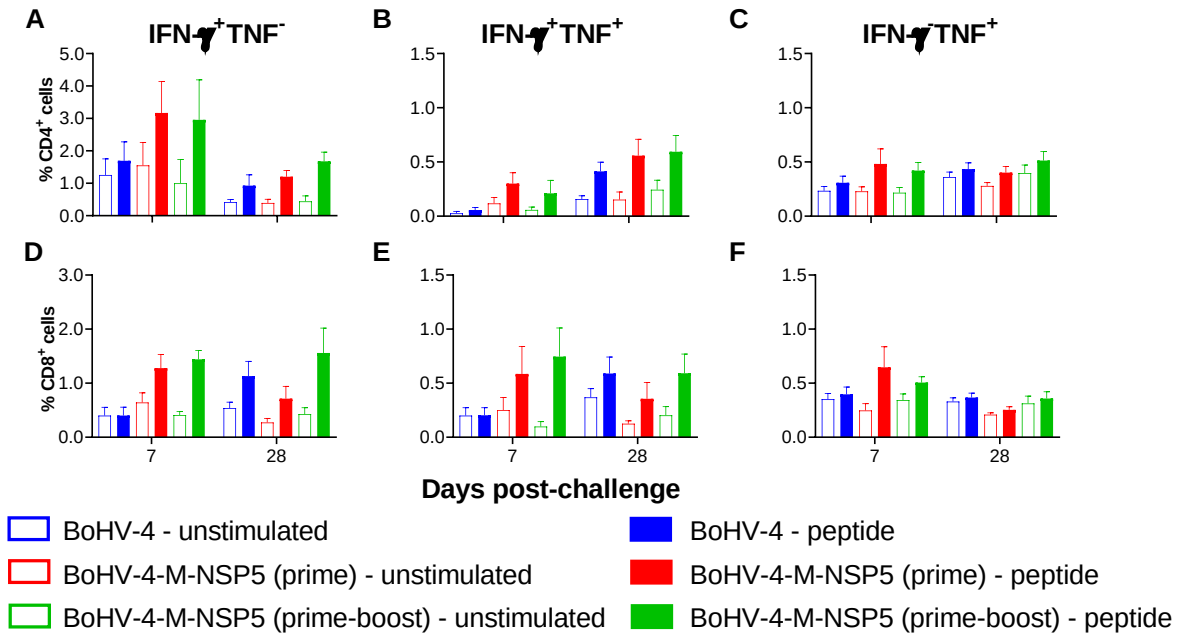
Supplementary Figure 12. Quantification of porcine IL10 in supernatant of peptide stimulated PBMC. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (study 1). Control pigs were immunized twice with the BoHV-4 vector before PRRSV challenge. IL10 in supernatant of culture of cryopreserved PBMCs from D21, D42 and D70 were assessed following with and without peptide stimulation by ELISA. Mean IL-10 concentrations ± SD are presented for each vaccine group.



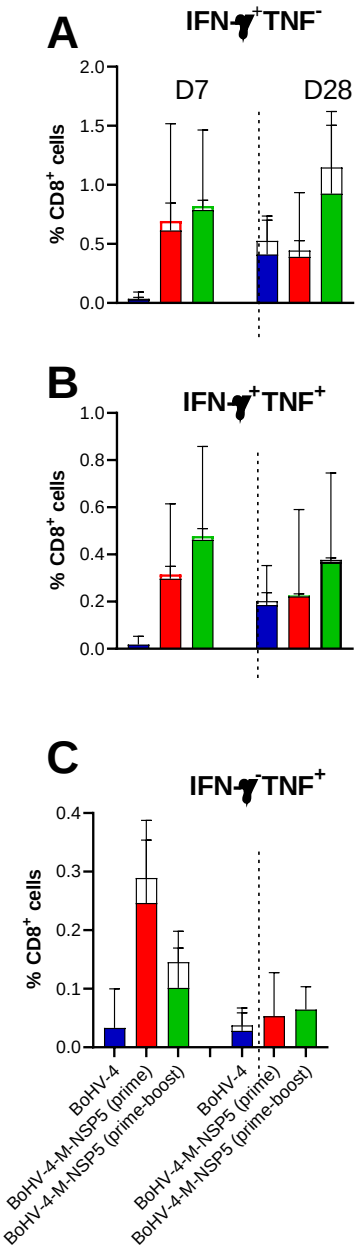
Supplementary Figure 13. Flow cytometric gating strategy for T cell phenotyping of bronchoalveolar lavage cells. Cells were gated by their characteristic light scattering and then further gated to exclude dead cells. Live lymphocytes were separated into $CD3^+$ T-cells and $CD3^-$ non-T-cells before $CD3^+$ cells were sub-gated for $CD4^+$ T cells, $CD8\alpha^{high}$ T cells, non-conventional T cells defined as $CD3^+$ $CD8\alpha^{-/low}$ $CD4^-$ T-cells. The proportion of these cell populations expressing CD25 was then assessed. Tregs were defined at $CD3^+$ $CD4^+$ $CD25^{high}$ $FoxP3^+$.



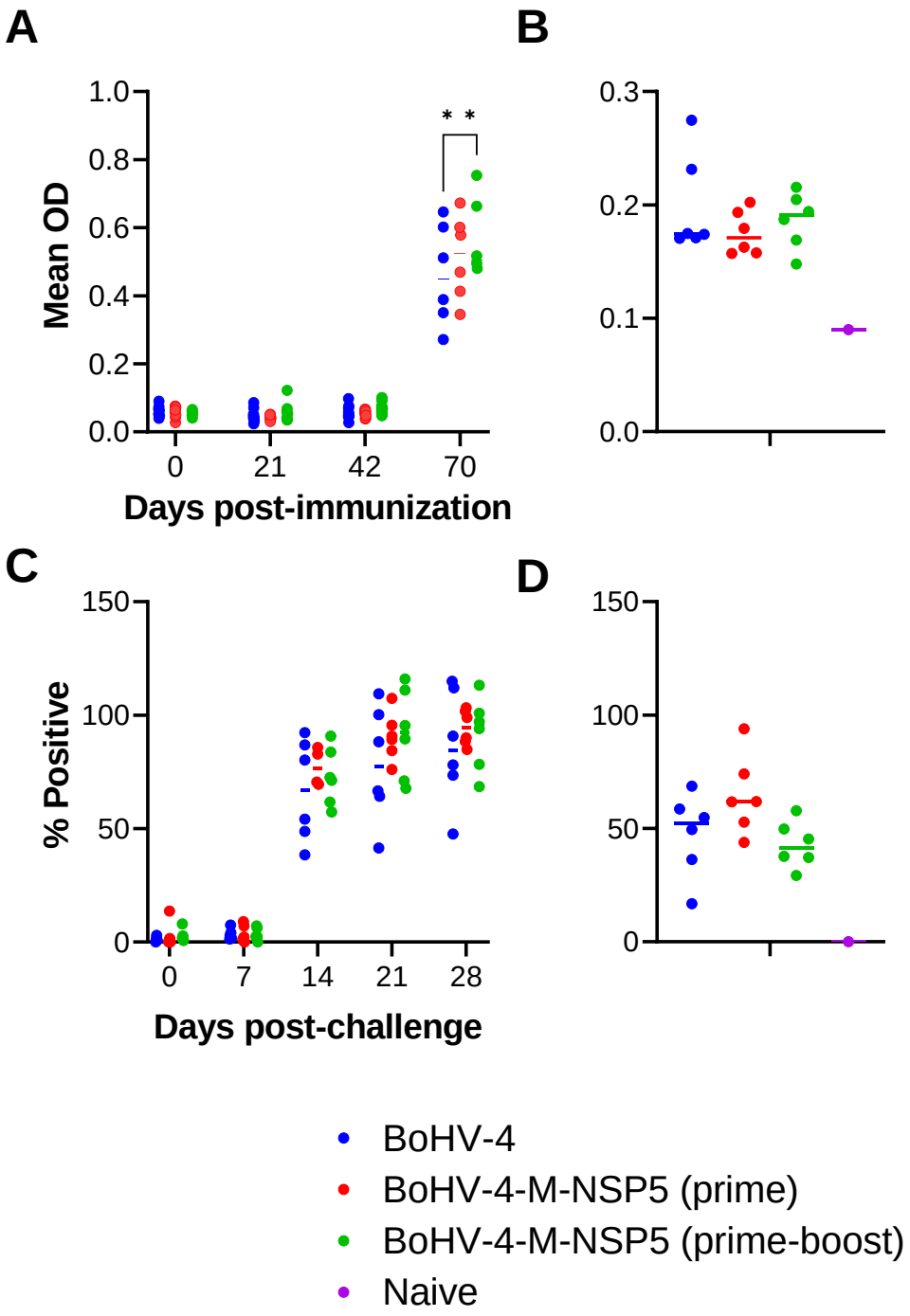
Supplementary Figure 14. Phenotyping of T-cell subsets infiltrating the lungs following BoHV-4-M-NSP5 vaccination and PRRSV-1 challenge. Pigs were immunized once (prime) or twice (prime-boost) with BoHV-4-M-NSP5 or twice with the BoHV-4 vector before challenge with PRRSV-1 21301/19 (study 1). Following euthanasia on 7- and 28-days post-challenge, BALC were isolated. Previously cryopreserved cells were labelled and analyzed by flow cytometry. The proportion of $CD3^+$ T cells (**A**), $CD4^+$ T cells (**B**), $CD8^+$ T cells (**C**), non-conventional T cells (**D**) was assessed. The percentage of these cells expressing the activation marker CD25 was also assessed (**E-H**). The proportion of Tregs in BALC was also determined (**I**). Mean data ± SD for each vaccine group are shown with datapoints representing individual pigs.



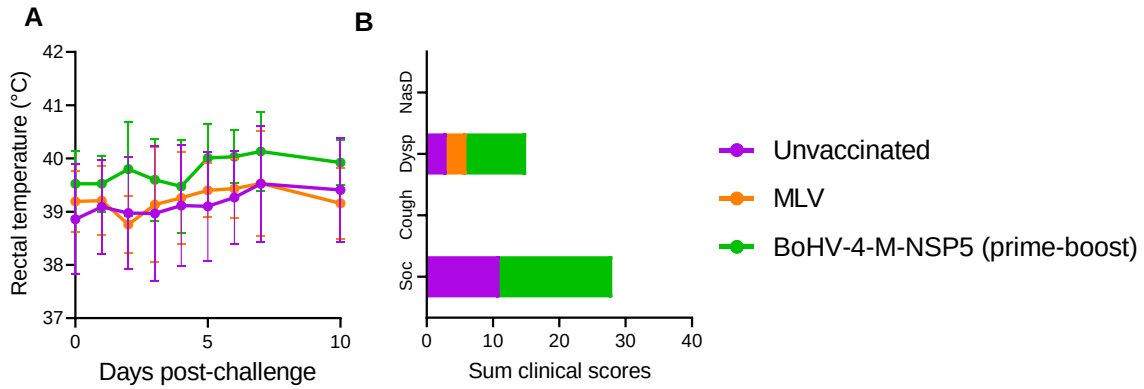
Supplementary Figure 15. Characterization of antigen-specific IFN-γ and TNF responses in the lungs of pigs after vaccination with BoHV-4-M-NSP5 and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-M-NSP5 and challenge with PRRSV-1 21301/19 (Study 1). Control pigs were immunized twice with the BoHV-4 vector before PRRSV challenge. Responses of previously cryopreserved BALC from 7- and 28-days post-challenge were assessed with and without peptide stimulation by flow cytometry. Expression of IFN-γ alone (**A**, **D**), IFN-γ and TNF (**B**, **E**), or TNF alone (**C**, **F**,) by CD4 T cells (**A**-**C**) and CD8 T cells (**D**-**F**) are shown as mean data ± SD for each vaccine group.



Supplementary Figure 16. Assessment of perforin expression by CD8 T cells after vaccination with BoHV-4-NSP5-M and PRRSV-1 challenge. PRRSV-1 M/NSP5 specific cytokine responses were assessed following one (prime) or two (prime-boost) immunizations of pigs with BoHV-4-NSP5-M and challenge with PRRSV-1 21301/19 (study 1). Control pigs were immunized twice with the BoHV-4 vector before challenge. Responses of previously cryopreserved BALC from 7- and 28-days post-challenge were assessed following peptide stimulation by flow cytometry. Perforin expression (perforin$^+$ - empty bars; perforin$^-$ - solid bars) by CD8 T cells expressing IFN-γ alone (**A**), IFN-γ and TNF (**B**), or TNF alone (**C**) are shown as mean unstimulated condition corrected data ± SD for each vaccine group.



Supplementary Figure 17. Assessment of antibody responses following BoHV-4-M-NSP5 vaccination and PRRSV-1 challenge. Pigs were immunized once (prime) or twice (prime-boost) with BoHV-4-M-NSP5 or twice with the BoHV-4 vector before challenge on D42 with PRRSV-1 21301/19 (study 1). An infected cell lysate was used in ELISA to assess PRRSV-1-specific antibodies in serum (**A**) and BALF (**B**) and a diagnostic ELISA was used to assess PRRSV N protein-specific antibodies in serum (**C**) and BALF (**D**). The results were expressed as OD_{450} or percentage of positivity (PP) according to the formula: PP = (OD_{450} sample − OD_{450} negative control / OD_{450} positive control − OD_{450} negative control) × 100. Datapoints represent individual pigs with the median indicated by a horizontal line. BALF from a PRRSV-naïve pig was included as an additional negative control.



Supplementary Figure 18. Evaluation of the efficacy of a BoHV-4 vectored PRRSV vaccine candidate. The ability BoHV-4-M-NSP5 immunization to confer clinical protection was assessed following challenge infection of pigs with PRRSV-1 LT-3 (Study 2). A negative control group comprised no immunization and a positive control group was immunized with PRRSV-1 MLV. Mean data ± SD for rectal temperatures over the course of challenge (**A**) is shown. The sum of clinical scores observed in the post-challenge period are presented for each group (**B**). Nasal discharge – NasD; dyspnoea – Dysp, and changes in social behavior – Soc.