A multi-epitope DNA vaccine co-administered with monophosphoryl lipid A adjuvant provides protection against tick transmitted *Ehrlichia ruminantium* in sheep

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Abstract

Previously, a heartwater experimental DNA vaccine provided 100% protection following laboratory challenge with *Ehrlichia ruminantium* administered by needle but not against an *E. ruminantium* tick challenge in the field. A multi-epitope DNA vaccine incorporating both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes epitopes could provide a better alternative. In this study, we investigated the use of multi-epitope DNA vaccines against an *E. ruminantium* experimental tick challenge in sheep. The multi-epitope DNA vaccines were delivered via the intramuscular route and intradermal route using the gene gun in the presence of monophosphoryl lipid A (MPL) adjuvant, which was either applied topically to the gene gun inoculation site or co-administered with the vaccine via the intramuscular route. Initially two constructs namely, pSignal plus and pLamp were tested with MPL applied topically only and no protection was obtained in this formulation. However, when the pLamp was co-administered with MPL via the intramuscular route in addition to topical application, its protective efficiency improved to protect 60% of the sheep against tick challenge. In this formulation, the vaccine induced enhanced activation of memory T cell responses both before and after challenge with variations amongst the different sheep possibly due to their different genetic backgrounds. In conclusion, this study showed that a heartwater multi-epitope DNA vaccine, co-administered with MPL adjuvant can protect sheep following a laboratory *E. ruminantium* tick challenge.

Keywords: Multi-epitope DNA vaccine, monophosphoryl lipid A, *E. ruminantium*, tick challenge

Highlights

- *E. ruminantium* peptides formulated in multi-epitope DNA vaccine constructs induced Th1 immunity *in vitro*
- pLamp multi-epitope DNA vaccine construct induced improved Th1 immune responses
- Co-administration of pLamp with MPL adjuvant protected 60% of sheep against *E. ruminantium* tick challenge

1. Introduction

Currently, there is no safe vaccine for heartwater. Recombinant vaccines are considered better alternatives to the live blood, attenuated and inactivated heartwater vaccines which are associated with safety concerns [1]. It is well documented that a cellular Th1 immune response is crucial in the protection against heartwater. These responses are mediated by $CD8^+$ and $CD4^+$ T lymphocytes through the production of the Th1 cytokine IFN-γ [2]. A successful heartwater DNA vaccine might result from multi-epitope DNA vaccines that can elicit similar immune responses. Previously, we identified $CD4^+$ T cell and $CD8^+$ cytotoxic T lymphocyte (CTL) epitopes from the following *E. ruminantium* antigens: Erum2540; Erum7140; Erum7320; Erum7350; Erum7620; and Erum8010 [3,4]. Four of the epitopes induced positive CTL responses, proliferation of $CD8^+$ T cells as well as production of IFN-γ by these cells [3]. Eight could induce IFN-γ production by memory $CD4^+$ T cells in addition to expression of other Th1 cytokines [4]. In the current study, these epitopes were used to construct different multi-epitope DNA vaccines.

A multi-epitope DNA vaccine should consist of $CD8^+$ and $CD4^+$ T cell epitopes in order to induce robust protective immune responses. Several multi-epitope DNA vaccines have been formulated in attempts to induce the activation of both $CD4^+$ Th1 and CTL responses. These include vaccines against pathogens like Hepatitis C virus [5], *Leishmania* [6], and *Toxoplasma gondii* [7]. These vaccines increased epitope processing and presentation to T-lymphocytes and elicited broad, high magnitude $CD4^+$ and $CD8^+$ T cell responses. Additionally, the construction of multi-epitope DNA vaccines and their immunogenicity depends on several factors including the influence of spacers between the epitopes. The addition of spacers has been shown to be effective in accurate processing of epitopes and recognition by T cells [8,9]. For example the addition of spacer residues AYY between epitopes of human papillomavirus type 16 resulted in increased CTL immune responses [8]. Target sequences, for example ubiquitin (UB), can be added to the vaccine vector to target antigens to a cellular compartment for degradation, enhancing cytotoxic T cell immunogenicity [10].

The innate immune system recognises specific molecular structures present in the pathogen in order to activate adaptive immunity [11]. As subunit vaccines, DNA vaccines lack the molecular structures that are found in live or attenuated vaccines. Hence, they are often unable to stimulate pathogen-specific adaptive immune responses and have to rely on the incorporation of effective adjuvants to enhance their immunogenicity [12,13]. Adjuvants like monophosphoryl lipid A (MPL) activate innate immune pathways that aid in the enhancement of adaptive immune responses [14]. Therefore, the use of MPL adjuvant, a derivative of lipopolysaccharide, activates toll like receptor (TLR) 4, which is one of the innate receptors resulting in activation of multiple innate functions that will support activation of adaptive immune responses [15,16].

In this study, two multi-epitope DNA vaccines (pSignal plus and pLamp) were constructed and their efficacy was tested *in vitro* to determine if they express the epitopes and induce similar immune response as determined for the synthetic peptides. Thereafter, the constructs were tested in sheep against heartwater challenge using *E. ruminantium* infected ticks. Cellular immune responses induced after immunisation and challenge were also studied.

2. Materials and Methods

2.1. Immune responses induced in vitro *by peptide pools*

Previously characterised $CD4^+$ and $CD8^+$ CTL epitopes (Supplementary Table 1) were combined in two pools in order to exclude peptide combinations in the vaccine that could have potential inhibitory effects. Pool 1 comprised of p7140-12, p7140-13, p7620-2, p8010-8, p7320-8, p7320-9, p2540-6, p2540-16, p2540-19 and p2540-20. Pool 2 consisted of p7140-6, p7140-7, p7140-20, p7620-12, p8010-8, p7320-21, p7350-9, p2540-21, p2540-6, p2540-16, p2540-19 and p2540-20. The ability of the peptide pools to induce cellular immune responses *in vitro* was tested using heartwater immune PBMC from infected and challenged three merino sheep (6821, 6822 and 6823) [17]. The peptide pools were tested for their ability to induce IFN-γ production using ELISpot assay and expression of other cytokines (IL-1α, GM-CSF, TNF-α, iNOS, IL-10) using quantitative real-time PCR (Supplementary methods 1).

2.2. Construction of the multi-epitope DNA vaccines

Two DNA vaccine constructs derived from twelve sheep codon optimised nucleotide sequences of *E. ruminantium* CD4 (p2540-21, p7140-6, p7140-7, p7140-20, p7320-21, p7350-9, p7620-12 and p8010-8), and CD8 CTL (p2540-6, p2540-16, p2540-19 and p2540-20) peptides were engineered as follows. The first construct, pSignal plus contained sheep CXCL1 signal sequence [18], a MHC II targeting sequence (LAMP sequence, RRKSYAGYQTL) [19] followed by eight CD4 epitopes with GPGPG spacers in between [20] and a CpG motif (CpG2135) [21]. This was followed by an IRES and an UB signal [10] before the four CD8 epitopes with AYY spacers in between [9] and a CpG2135 motif (Supplementary Figure 1A). The second construct, pLamp, was similar to pSignal plus but did not contain the CXCL1 signal sequence (Supplementary Figure 1B). The synthetic constructs (Genscript) were cloned into the NotI/XbaI restriction site of the pcDNA3.1 (+) vector (Invitrogen) and transformed into *E. coli* and recombinant clones were confirmed by DNA sequencing. For immunisation, plasmid DNA was prepared using the Endofree Plasmid Maxi Kit (Qiagen) following the manufacturer's instructions. The DNA concentration and purity was determined using a spectrophotometer

(ND-1000 Nanodrop®, Thermo Scientific). The plasmid DNA concentrations were adjusted to a final concentration of 1 µg/ml in PBS and stored at – 20°C until use.

2.3. Evaluation of immune responses induced in vitro *by the multi-epitope DNA vaccine constructs* Immune PBMC from heartwater immune sheep 6821, 6822 and 6823 [17] were electroporated with either empty vector for negative control or with either of the two plasmid DNA constructs (pSignal plus and pLamp) using the GenePulser Xcell (Bio-Rad). PBMC (1 x 10^7 cells/ml) were washed with PBS and resuspended in 1 ml electroporation buffer (Bio-Rad). Cells were divided into 250 µl aliquots and each aliquot of cells was electroporated with 4 µg of the empty vector or pLamp and pSignal at an optimised voltage setting of 300 V with constant capacitance at 1000 µF and ∞ Ω resistance [22]. Cells were gently resuspended in 1 ml of pre-warmed cRPMI-1640 (GIBCO® RPMI+GlutaMAXTM-I) (Invitrogen) medium supplemented with 10% foetal bovine serum (Life Technologies), 55 mM 2mercaptoethanol (Sigma) and 1% GIBCO® Pen Strep (Invitrogen). Cells were seeded in triplicate in a 48 well plate at 5 x 10^5 cells/well and were incubated for 72 h at 37°C in a humidified 5% CO_2 incubator. Immune responses induced *in vitro* by the DNA vaccine constructs were evaluated using flow cytometry. The phenotype of the cells activated by the DNA vaccine constructs was determined by cell surface staining while their ability to produce IFN-γ was determined by intracellular cytokine staining (Supplementary methods 2).

2.4. Preparation of plasmid DNA for gene gun inoculations

The plasmids were precipitated onto 1.6 µm gold particles (BioRad) and coated into Tefzel tubing (BioRad) before each inoculation according to the Helios Gene Gun system's (BioRad) instructions (Supplementary methods 3).

2.5. Immunisation and challenge of animals

2.5.1 Animals

Merino sheep (n=33) aged between 8 and 12 months were obtained from a heartwater free region (Warden, Free State Province) in South Africa and tested negative for *E. ruminantium* using the pCS20 qPCR [23]. Animals were housed in tick free stables at ARC-OVR. All animal research protocols were approved by the animal ethics committee at the ARC-OVR. This study was approved by the South African Department of Agriculture Forestry and Fisheries under section 20 of the Animal Diseases Act of 1984 with reference (12/11/1/1).

2.5.2 MHC typing of the experimental animals

Typing for Ovine MHC *Ovar*-DRB1 was performed using PCR-RFLP as described previously [17].

2.5.3 Animal trial 1

Four groups of sheep (n=5) (Supplementary Table 2) were immunised three times at three week intervals with: 1. Empty vector (pcDNA3.1(+) vector); 2. Empty vector + adjuvant; 3. pLamp + adjuvant; 4. pSignal Plus + adjuvant. The adjuvant, MPL from *Salmonella enterica* serovar Minnesota (L8695, Sigma) was prepared to a final concentration of 1 mg/ml in DMSO and stored at - 70°C until use. For inoculation, MPL was diluted in DMSO to 10 µg/20 µl, which was applied topically to the gene gun inoculation site. All the sheep received 200 µg DNA vaccine construct delivered by intramuscular injection [24] and 50 µg delivered intradermaly by the gene gun using the Helios Gene Gun system (BioRad) with helium pressure of 300 psi. Additional control groups were the naïve sheep (n=2) and the tick immunised positive control (n=2); which were tick infected with *E. ruminantium* Welgevonden strain as described previously [17] and treated with Terramycin®100 (1 ml/10 kg) on the third day of febrile reaction. Five weeks after the third DNA inoculation, all the sheep were challenged with *E. ruminantium* infected adult ticks. Briefly, an area on the back of each sheep was shaved and a bag was attached to the shaven area. Five *E. ruminantium* infected male ticks were added to the bag and allowed to feed for at least three days. After this, five *E. ruminantium* infected female ticks were added

to the bag and allowed to feed until engorged. The sheep were monitored for the onset of clinical symptoms and rectal temperatures were measured daily. To determine the severity of infection, clinical signs were scored using a reaction index (RI) as described previously [24]. Animals with body temperatures of 42°C combined with any of the following symptoms: loss of appetite, heavy breathing, depression, hanging head, stiff gait, exaggerated blinking, chewing movements, anorexia and signs of nervous symptoms were treated with Terramycin®100 (1 ml/10 kg). These animals were regarded as non-survivors.

2.5.4 Animal trial 2

In addition to topical application at the gene gun inoculation site, the adjuvant was also co-administered with pLamp DNA vaccine via intramuscular route. Briefly, 40 µg of adjuvant was mixed with 200 µg DNA construct per sheep and a total volume of 2 ml was administered intramuscularly. Two groups of sheep (n=5, Supplementary Table 2) were immunised three times at three weeks intervals with: 1. Empty vector co-administered with adjuvant; 2. pLamp co-administered with adjuvant. Sheep received 200 µg DNA construct co-administered with adjuvant by intramuscular injection and 50 µg delivered intradermal as above. Control groups were the non-immunised naïve sheep (n=2) and positive control (n=2) tick infected and treated sheep as described above. Five weeks after the third DNA inoculation, all the sheep were tick challenged with *E. ruminantium* infected adult ticks and monitored as described above.

2.6. Cellular immune responses induced following immunisation and challenge

ELISpot assay and cell surface staining were performed in order to evaluate cellular immune responses induced following vaccination and challenge. Sheep were bled before immunisation, before challenge as well as on days 13, 15, 16, and 20 after challenge. PBMC were isolated from whole blood and stimulated with a cocktail of appropriate *E. ruminantium* peptides with total peptide concentration of 50 µg/ml. ELISpot assay was performed in triplicate wells using the Bovine IFN-γ ELISpot^{PLUS} kit (Mabtech) according to the

manufacturer's instructions. Following stimulation, cell surface staining was performed as described previously [3,4].

2.7. Statistical analysis

The significance of differences between the RI scores and ELISpot assay results were determined by means of the Student's *t*-test. Differences were considered significant at a p value of ≤ 0.05.

3. Results

3.1 Immune responses induced in vitro *by peptide pools*

In order to determine the best combination of epitopes for inclusion in multi-epitope DNA vaccines, previously characterised $CD4^+$ and $CD8^+$ CTL epitopes were pooled and immune responses induced *in vitro* were evaluated using PBMC from immune sheep 6821, 6822 and 6823. The ELISpot assay showed that, Pool 2 induced significant increase in IFN-γ production in PBMC from all three sheep (Table 1). However, Pool 1 induced less IFN-γ in PBMC from sheep 6821 and 6822. In addition to IFNγ, expression of other cytokines induced by Pool 1 and Pool 2 was studied by RT-qPCR assay (Table 1) using PBMC from sheep 6821 and 6823. Both pools induced the upregulation of IL-1α, GM-CSF, iNOS and TNF-α mRNA in PBMC from sheep 6821. Pool 1 induced a slight increase in the expression of IL-10 mRNA. Similarly, when the PBMC from sheep 6823 were used, all peptide pools were able to induce the upregulation of all cytokines tested except TNF-α and mRNA fold increase (FI) varied among housekeeping genes. Taken together the results obtained from the assays, Pool 2 was selected for inclusion in multi-epitope DNA vaccines.

3.2. Construction of multi-epitope DNA vaccines and evaluation of immune responses induced in vitro

Epitopes from peptide Pool 2 were engineered into synthetic genes, cloned in the pcDNA3.1(+) vector and sequence analysis confirmed that the DNA vaccine constructs contained the correct sequences and inserts. Immune responses induced *in vitro* by pLamp and pSignal plus were examined and flow cytometric analysis revealed that both induced significant percentage IFN-γ producing $CD4^+$ and $CD8^+$ T cells in comparison to the empty vector (Fig 1A-B) but this varied among animals. Similarly, pLamp and pSignal plus both induced much higher percentages of memory $CD4^+$ and $CD8^+$ T cells compared to the empty vector (Fig 1C-D).

3.3. MHC typing of the experimental animals

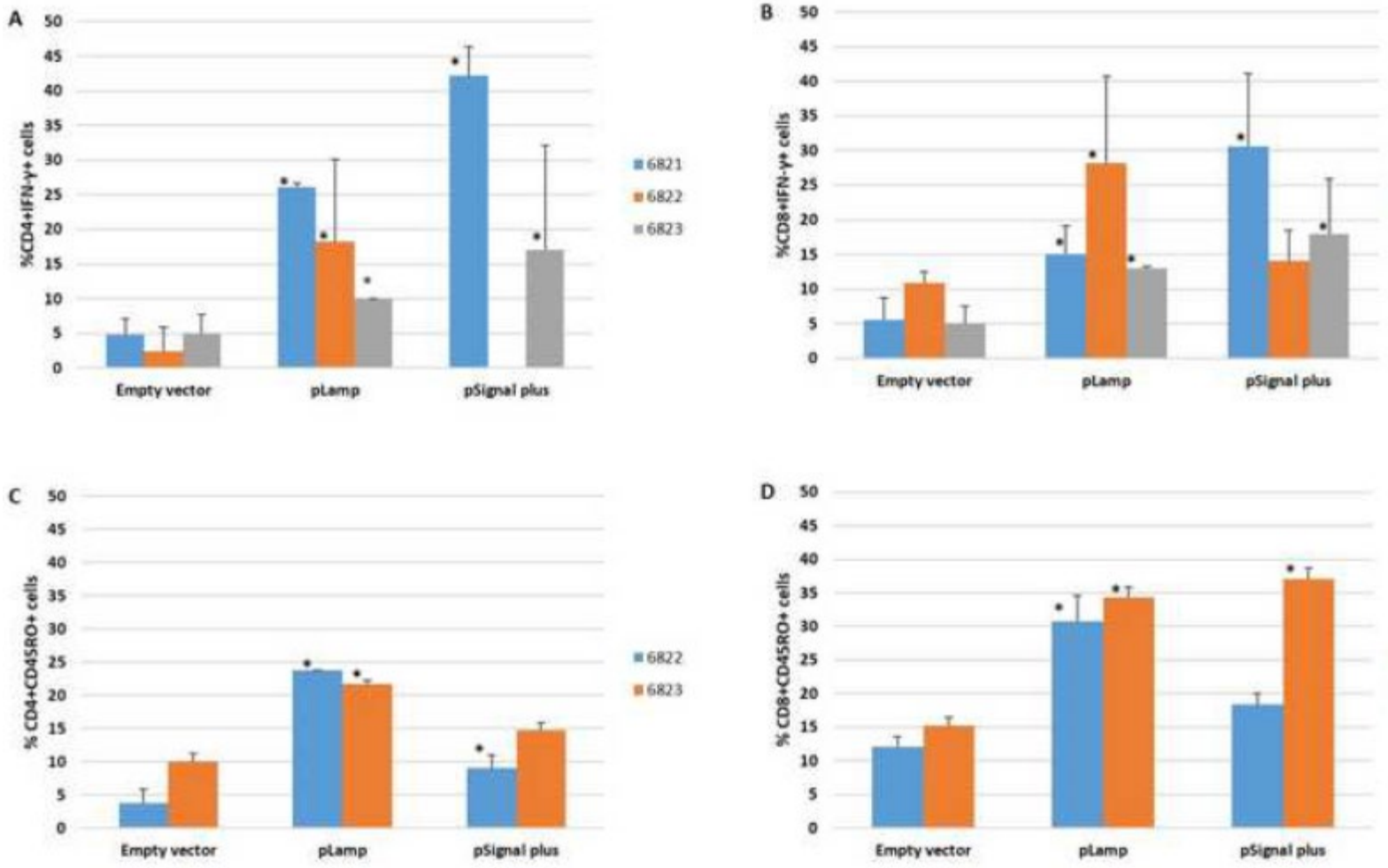
In order to ensure an even distribution of MHC class II DRB1 second exon (Ovar-DRB1) alleles between the animal groups in the vaccine trials, MHC typing was performed for all our experimental animals and the results

Table 1. Cytokine profiles induced by peptide pools in PBMC from immune sheep (6821, 6822 and 6823) 536 determined by ELISPOT and RT-qPCR assays. The PBMC were stimulated with peptide pools at a final total 537 concentration of 50 µg/ml.

			RT- qPCR: average relative mRNA FI[b] normalised to β-GAPDH after stimulation with peptide pools and actin (β-actin; GAPDH)				
Sheep	**Peptide pools**	**ELISpot (Ave Spmc ± SD)[a]; (P-value)**	**IL-1α**	**GM-CSF**	**iNOS**	**TNF-α**	**IL-10**
6821	Pool 1	**13±4 (0.017)**	2; 1	3; 2	2; 1	3; 2	3; 2
	Pool 2	**65±3 (0.001)**	3; 2	6; 3	4; 2	6; 3	1; 1
	Ag+	**90±4 (0.001)**	1; 1	1; 1	5; 7	1; 2	3; 5
6822	Pool 1	**53±10 (0.041)**	nd	nd	nd	nd	nd
	Pool 2	**90±9 (0.005)**	nd	nd	nd	nd	nd
	Ag+	**167±12 (0.005)**	nd	nd	nd	nd	nd
6823	Pool 1	2±2	3; 3	2; 2	2; 1	1; 1	2; 2
	Pool 2	**78±24 (0.036)**	4; 1	1; 3	5; 1	1; -[c]	2; -
	Ag+	**125±3 (0.003)**	1; -	-	-; 1	1; -	2; -

[a] Average number of spots/million PBMC for sheep ± the standard deviation for the experimental repeats. Bold values were statistically significant, p value ≤ 0.05. [b] Values represent the ΔΔCt values, calculated as the mean of duplicate samples each normalised to its own reference genes. Values ≥ 1 were considered as positive. The first value normalised to β-actin and the second value normalised to GAPDH. [c] (–) value less than one fold. nd- not done

Figure 1. Flow cytometric analysis of immune response induced in vitro by pLamp and pSignal plus compared to the empty vector. Percentage of cells expressing CD4 +IFN-γ+ (A), CD8+IFN-γ+ (B), CD4+CD45RO+ (C) and CD8+CD45RO+ (D) after PBMC were electroporated with the pDNA vaccine constructs in vitro. Representative data from sheep (6821, 6822 and 6823) are shown. Only the percentages that were two times higher than the percentage of the negative control and had significant p values ($p \leq 0.05$ as determined by Student's t-test) were regarded as positive and indicated as "*" on the graph. Error bars represent the standard deviation.



are summarised in Table 2. In trial 1: sheep 3321, 3315 and 3318 had a similar allele *03411, while sheep 3300, 3338 and 3314 also shared a similar allele *0201. Two different alleles (*0323 and *0333) were obtained for sheep 3304, 3311, 3335 and 3323. Additionally sheep 3311 also had allele *0332 and a new unknown allele. In trial 2: sheep 3499, 3320, and 3488 shared a similar allele *0203 (Table 2). Two sets of two different alleles (*0702 and *0703; *0323 and *0333) were obtained for sheep 3498 and 3299 respectively. Additionally, allele *0605 was obtained for sheep 3299. There were 11 new unknown alleles that were either shared amongst the different sheep or were found in different individual sheep. From the MHC typing results, animals were divided amongst the experimental groups in such a way that each MHC type was represented in each group where possible.

3.4. Animal trial 1: Protective efficiency of multi-epitope DNA vaccines following tick challenge

Following immunisation with the pLamp and pSignal plus multi-epitope vaccines, none of the sheep survived challenge (Table 2). These animals showed critical temperature increases between 14 and 20 days post challenge (dpc). This was accompanied by severe heartwater symptoms as shown by higher RI values (Fig 2A). Sheep inoculated with pLamp, had RI values of between 66 and 72 (Ave 68 ± 5.0) while those immunised with pSignal plus had higher RI values of between 66 and 83 (Ave 73 ± 8.0). Additionally, there was no significant RI differences between animals in these groups and their respective negative controls. All the sheep in the negative control and naïve groups succumbed to infection. The animals inoculated with the empty vector only, started showing temperature increases 14 and 16 dpc. While the sheep that received the empty vector with the adjuvant started showing critical temperature increases between 16 and 23 dpc. Only the sheep in the positive control group survived challenge.

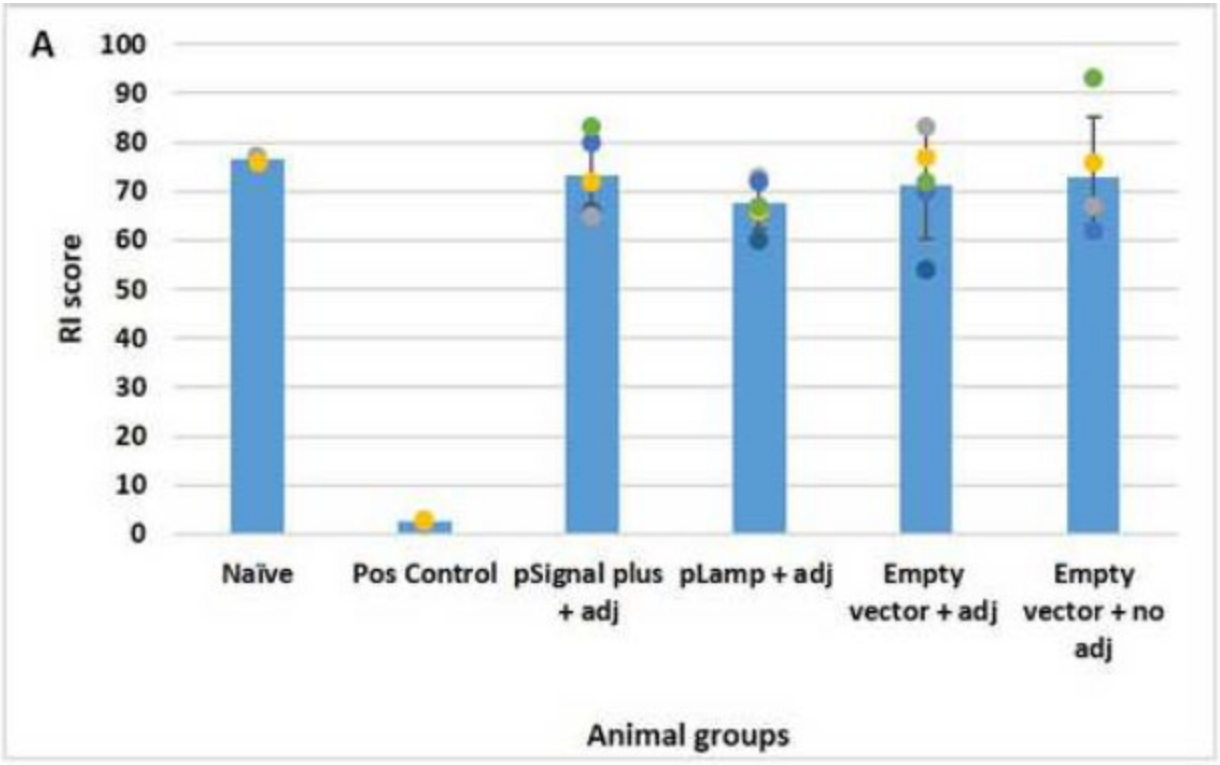
3.5. Animal trial 1: Ag-specific immune responses to vaccination with multi-epitope DNA vaccine constructs following tick challenge

T cell responses induced by the multi-epitope DNA vaccines was determined before challenge as well as 13 and 16 dpc using ELISpot assay (Table 3). When sheep were immunised with pLamp, only sheep 3297 (53±2.1)

Table 2. Animal trial 1 and 2. MHC typing of the experimental animals and summary of the survival of 561 immunised sheep following tick challenge.

Treatment	Sheep number	MHC DRB1 alleles	Days to temperature above 40˚C	Highest temperature reached (˚C)	Survival (S) or treatment (T) or dead (D) on day shown
		Animal trial 1			
Empty vector	3313	Unknown 6	14	42.0	T (19)
	3310	Unknown 7	14	41.8	T (17)
	3311	*0323, *0333; *0332, Unknown	16	42.0	T (21)
	3331	Unknown 2	14	41.9	D (20)
	3330	Unknown 6	16	40.1	T (19)
Empty vector + adjuvant (applied topical)	3295	Unknown 1	16	42.0	T (20)
	3321	*03411	19	41.5	T (22)
	3304	*0323; *0333	19	42.0	D (21)
	3329	Unknown 2	16	42.0	T (20)
	3300	*0201	23	41.6	T (23)
pLamp + adjuvant (applied topical)	3297	Unknown 1	15	42.0	T (19)
	3315	*03411	18	41.8	T (20)
	3335	*0323; *0333	16	42.0	T (19)
	3328	Unknown 2	14	42.0	T (18)
	3338	*0201	20	41.9	T (21)
pSignal plus + adjuvant (applied topical)	3303	Unknown 1	19	41.7	T (21)
	3318	*03411	17	42.0	T (20)
	3323	*0323; *0333	20	41.5	T
	3327	Unknown 2	16	42.0	T (19)
	3314	*0201	17	41.0	T (22)
Naive	3308	Unknown 5	19	42.0	T (22)
	3317	Unknown 3	15	42.0	T (18)
Positive control	3291	Unknown 8	-	39.6	S
	3292	Unknown 9	-	39.8	S
		Animal trial 2			
Empty vector co-administered with adjuvant	3499	*0203	18	41.9	T (20)
	3320	*0203	20	41.0	T (21)
pLamp co-administered with adjuvant	3299	*0323,*0333; *0605	16	42.0	T (20)
	3488	*0203	-	39.3	S
	3333	Unknown 4	16	41.8	T (20)
	3498	*0702; *0703 and Unknown	-	39.6	S
	3337	Unknown 5	-	39.0	S
Naive	3326	Unknown10	21	40.0	T (42)
	3336	Unknown11	20	41.9	T (22)

Figure 2. Average RI score of sheep immunised with pSignal plus and pLamp. Sheep were immunised with pDNA using both intramuscular injection and intradermal inoculation with the gene gun. In trial 1 (A) the adjuvant was applied topically only at the gene gun inoculation site and trial 2 (B) the adjuvant was applied topically at the gene gun inoculation site and co-administered with pDNA via intramuscular inoculation. Sheep were challenged with E. ruminantium infected ticks. The coloured dots represent the RI score of each sheep in the group. Error bars represent the standard deviation.



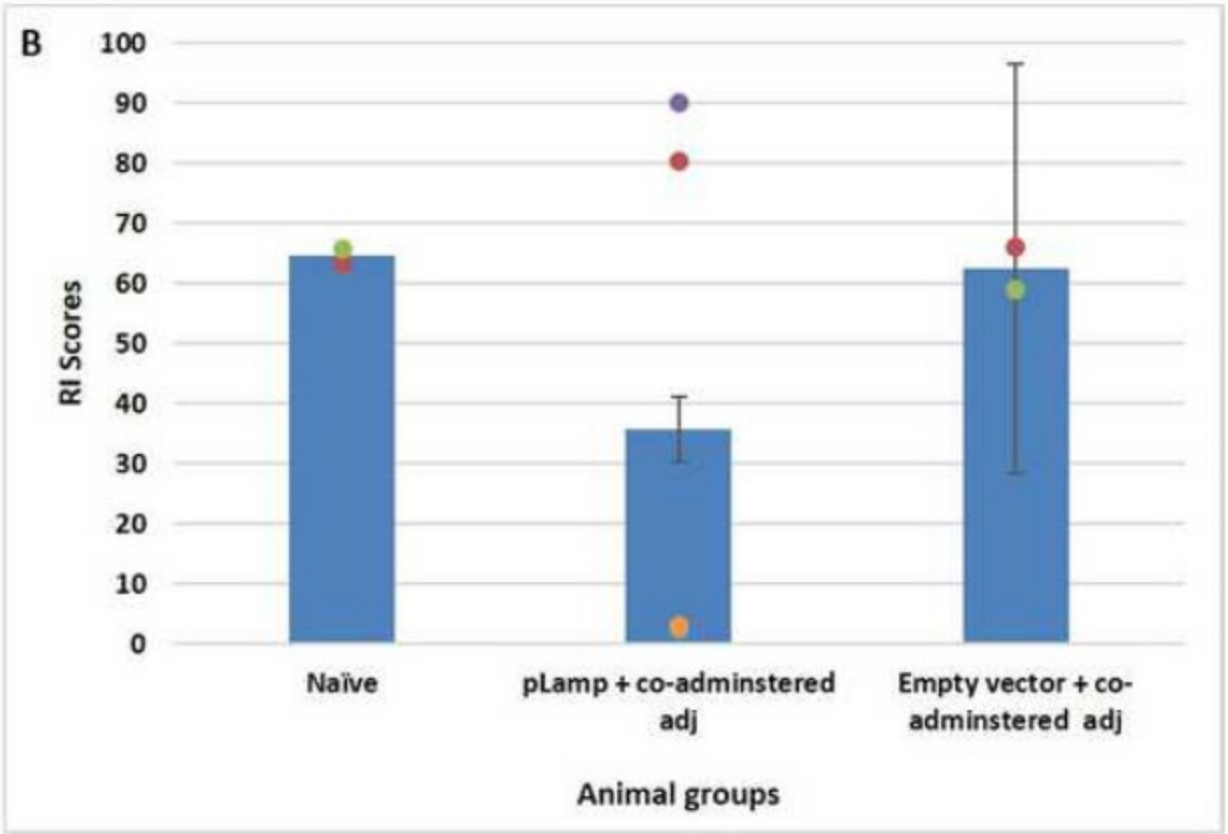


Table 3. Ag-specific IFN-γ responses from sheep immunised with different multi-epitope DNA vaccine constructs. Responses were determined before challenge, 13, 15, 16 and 20 days after tick challenge.

Treatment	Sheep number	Number of Spmc[a]					
		Before challenge	AVE: Before challenge	13/15 days after challenge	AVE: 13/15 days after challenge	16/20 days after challenge	AVE: 16/20 after challenge
Animal trial 1							
Empty vector + adjuvant topically	3295	58 ± 1.4		10 ± 3.5		88 ± 8.5	
	3321	28 ± 2.8		5 ± 0.7		58 ± 2.1	
	3304	25 ± 1.4	25±21.3[b]	23 ± 1.4	10±8.7[b]	5 ± 2.8	62±39.2[b]
	3329	0		0		108 ± 9.9	
	3300	15 ± 3.5		13 ± 5.7		53 ± 2.8	
pLamp + adjuvant topically	3297	**53 ± 2.1* (0.013)**		0		**255 ± 19.8* (0.034)**	
	3315	25 ± 4.2		**38 ± 0.0* (0.047)**		**625 ± 14.1* (0.026)**	
	3335	0		**93 ± 0.7* (0.0003)**		8 ± 3.5	
	3328	22.5 ± 2.8		**45 ± 8.5* (0.031)**		**133 ± 1.4* (0.005)**	
	3338	0		7.5 ± 3.5		0	
pSignal plus + adjuvant topically	3303	45 ± 2.1		3 ± 3.5		25 ± 5.7	
	3318	**60 ± 1.4* (0.006)**		**28 ± 2.1* (0.046)**		113 ± 12.7	
	3323	10 ± 4.2		8 ± 0.7		13 ± 2.8	
	3327	20 ± 4.9		0		48 ± 7.1	
	3314	0		0		93 ± 6.4	
Animal trial 2							
Empty vector + adjuvant topically & intramuscular	3499	10 ± 1.4	35±35.4	68 ± 6.4	64±6.7	nd	
	3320	60 ± 17		60 ± 9.9		98 ± 5.7	
pLamp + adjuvant topically & intramuscular	3299	50 ± 6.4		5 ± 4.2		nd	
	3488	25 ± 3.5		38 ± 3.5		30 ± 3.5	
	3333	33 ± 5.7		0		nd	
	3498	15 ± 2.1		**145 ± 9.9 (0.028)**		40 ± 2.8	
	3337	20 ± 0.4		60 ± 1.4		15 ± 2.8	

[a]The number of IFN-γ producing cells were expressed as Spmc. Ag-specific Spmc of the immunised sheep were compared to the average Spmc of the empty vector group. Only Ag-specific Spmc that were twice or more than the average Spmc of the empty vector group and had significant *p* values (* $p \leq 0.05$ as determined by Student *t test*) were regarded as a positive and these are represented in bold. [b]The average Spmc for the empty vector group. nd = not determined.

showed significant IFN-γ production before tick challenge when compared to the average responses in the empty vector group. Thirteen dpc, sheep 3315 (38±0.0), 3335 (93±0.7) and 3328 (45±8.5) showed significant IFN-γ responses. Sheep 3297 (255±19.8), 3315 (625±14.1) and 3328 (133±1.4) showed significant number of IFN-γ producing cells 16 dpc. When sheep were immunised with pSignal plus, only sheep 3318 showed significant Ag-specific IFN-γ production before challenge (60±1.4) and 13 dpc (28±2.1). However, 16 dpc, there was no significant IFN-γ production observed in the sheep in this group compared to the empty vector group. The number of memory $CD4^+$ and $CD8^+$ T cells were also measured by flow cytometry before and post tick challenge (Table 4). In the pLamp group, only sheep 3338 showed an increase in the number of memory $CD4^+$ T cells (19%) before challenge in comparison to the empty vector group. Thirteen dpc, sheep 3315 and 3328 showed 5% and 2% increase respectively while 16 dpc no difference was observed. When the number of memory $CD8^+$ T cells were measured before challenge, sheep 3335 (23%) and 3338 (19%) showed an increase compared to the empty vector group. Thirteen dpc, sheep 3315 and 3335 showed 37% and 16% respectively while 16 dpc no increase was obtained. Sheep that were immunised with the pSignal plus construct showed no induction of memory $CD4^+$ and $CD8^+$ T cells before challenge as well as 13 dpc compared to sheep inoculated with the empty vector. However, 16 dpc, sheep 3314 (55%) and sheep 3327 (43%) showed an increase only in the number of memory $CD8^+$ T cells.

3.6. Animal trial 2: Protective efficiency of pLamp co-administered with adjuvant

Since animals immunised with pLamp had slightly lower RI values with higher immune responses, this construct was chosen for the second animal trial where MPL was co-administered with the DNA vaccine via intramuscular immunisation in addition to the topical application at the gene gun inoculation site. This resulted in three (3488, 3498 and 3337) of the five sheep surviving a tick challenge (Table 2). The two sheep (3299 and 3333) that succumbed to infection had increased body temperature as well as severe heartwater symptoms as shown by RI values of 80 and 90 respectively. On the other hand, the three sheep (3488, 3498 and 3337) that survived showed no temperature increases nor heartwater symptoms as shown by RI of 3 for each animal (Fig2B, Table 2). All the sheep in the negative control and naïve groups succumbed to the disease.

Table 4. Percentages of memory $CD4^+$ and $CD8^+$ T cells from sheep immunised with different DNA vaccine constructs. Responses were determined before challenge, 13, 15, 16 and 20 days after tick challenge.

Treatment	Sheep number	% of $CD4^+$ $CD45RO^+$						% of $CD8^+$ $CD45RO^+$					
		Before challenge	**AVE: before challenge**	**13/15 days after challenge**	**AVE: 13/15 dpc**	**16/20 days after challenge**	**AVE: 16/20 dpc**	**Before challenge**	**AVE: before challenge**	**13/15 days after challenge**	**AVE: 13/15 dpc**	**16/20 days after challenge**	**AVE: 16/20 dpc**
Animal trial 1													
Empty vector + adjuvant topically	3295	0		0		2				14		13	
	3321	0		0		0		14		0		9	
	3304	0	0	0	0	15	8±6.3	7	9±10.9	0	4±6.2	30	16±10.9
	3329	0		0		5		0		7		23	
pLamp + adjuvant topically	3300	0		0		0		26		0		3	
	3297	0		0		0		0		5		0	
	3315	0		**5**		5		6		**37**		27	
	3335	0	**4±8.5**	0	1±2.2	0	1±2.2	**23**	10±10.8	**16**	12±15.6	16	9±12.4
	3328	0		**2**		0		0		0		0	
	3338	**19**		0		0		**19**		0		0	
pSignal plus + adjuvant topically	3303	0		0		0		0		0		0	
	3318	0		0		0		0		0		18	
	3323	0		0		0		9		0		31	
	3327	0		0		0		0		1		**43**	
	3314	0		0		13		0		0		**55**	
Animal trial 2													
Empty vector + adjuvant topically & co-administered IM	3499	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	3320	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
pLamp + adjuvant topically & co-administered IM	3299	3		38		nd		5		50		nd	
	3488	6		0		1		37		10		3	

3333	0	10±10.8	18	13±15.7	nd	7±10.7	30	35±20.8	55	31±21.5	nd	18±29.2
3498	4		0		0		63		30		0	
3337	0		11		19		39		9		52	

Animal trial 1: Percentage of memory $CD4^+$ and $CD8^+$ T cells of sheep immunised with pLamp or pSignal plus were compared to the average percentage of $CD4^+$ and $CD8^+$ T cells of the empty vector group. Only values that were twice or higher than the average percentage for empty vector group were regarded as positive and these are represented in bold. Animal trial 2: Average percentage of memory $CD4^+$ and $CD8^+$ T cells of sheep immunised with pLamp co-administered IM with adjuvant were compared to the average percentage of memory $CD4^+$ and $CD8^+$ T cells of sheep immunised using pLamp with adjuvant applied topically only. nd = not determined.

3.7. Animal trial 2: Ag-specific immune responses to vaccination with pLamp following tick challenge

Ag-specific IFN-γ responses (Table 3) and the number of memory $CD4^+$ and $CD8^+$ T cells (Table 4) were measured before challenge as well as 15 and 20 dpc. When sheep were immunised with pLamp co-administered with adjuvant topically and intramuscularly, only sheep 3498 showed a significant number of IFN- γ producing cells (145±9.9) 15 dpc when compared to the empty vector group. The number of memory $CD4^+$ and $CD8^+$ cells of sheep immunised with pLamp co-administered intramuscularly with MPL was compared to that of sheep immunised with pLamp with adjuvant applied topically only (trial 1). In individual sheep (Table 4), the number of these cells varied amongst the different animals, which was expected as this, were outbred animals. On average, the number of memory $CD4^+$ T cells in the two groups were not very different (4±8.5 and 3±2.6) before challenge. After challenge, the average number of these cells were higher in animals immunised with pLamp co-administered intramuscularly with adjuvant showing an average of 13±15.74 (15 dpc) and 7±10.7 (20 dpc) compared to an average of 1±2.2 (13 dpc) and 1±2.2 (15 dpc) when adjuvant was applied topically only. The average number of memory $CD8^+$ T cells before challenge was higher in the sheep that had the vaccine co-administered intramuscularly with the adjuvant (35±20.8) in comparison to the sheep that had the adjuvant applied topically (10±10.8). After challenge, the same was observed with the sheep that had the adjuvant co-administration with the vaccine showing an average of 31±20.5 (15 dpc) and18±29.2 (20 dpc) compared to the adjuvant applied topically, which showed an average of 12±15.6(13 dpc), and 9±12.4 (16 dpc).

4. Discussion

Previously, we showed that DNA vaccines could provide between 20% and 100% protection in sheep against laboratory *E. ruminantium* needle challenge [24,25]. However, the most effective 1H12 DNA vaccine failed to protect animals against field tick challenge [26]. This study was undertaken to investigate the use of multi-epitope DNA vaccines against heartwater. These vaccines, allow for the use of multiple epitopes targeted at different cells of the immune system. We have since identified $CD4^+$ T cell and $CD8^+$ CTL epitopes *in vitro* [3,4] which were used to construct two different multi-epitope DNA vaccines. Their protective efficiency was tested against heartwater challenge using *E. ruminantium* Welgevonden infected ticks.

To select the best combination of peptides for inclusion in the multi-epitope DNA vaccine, two peptide pool combinations were assessed for their ability to induce immune responses *in vitro*. Both pools induced IFN-γ production and upregulation of other Th1 cytokines with pool 2 inducing the best responses. These responses, although relatively low, corresponded with responses induced by the individual peptides [3,4]. Pool 2 with eight CD4 epitopes and four CD8 epitopes was thus used for construction of the two multi-epitope DNA vaccines (pSignal plus and pLamp). These constructs could induce production of IFN-γ by $CD4^+$ and $CD8^+$ T cells in addition to activation of memory $CD4^+$ and $CD8^+$ T cells *in vitro*, which correlated with responses induced by the synthetic peptides. This showed that the constructs expressed *E. ruminantium* peptides *in vitro* and these were successfully presented to T cells, resulting in activation of these cells.

In the first animal trial, pSignal plus and pLamp were tested in sheep for their protective efficacy against experimental tick challenge. When administered intramuscularly and intradermaly in the presence of MPL applied topically, none of the constructs could protect sheep against tick challenge. The pSignal plus construct contained an additional CXCL1 signal sequence for antigen export [18,27] and subsequent uptake by antigen presenting cells (APC) or recognition by B-cells [28], while the LAMP-antigen chimeras are directly targeted to the lysosome for association with MHC class II *in situ* [19,29]. However, animals immunised with the pSignal

construct had slightly higher RI values and were less effective at Th1 immune response induction than those immunised with pLamp, indicating that this signal sequence lowered the efficacy of this vaccine construct and should perhaps only be included with epitopes specific for B cells.

MPL is one of the adjuvants that is undesirable for transcutaneous immunisation or topical application due to its inability to cross the stratum corneum, which is often impermeable to water-soluble macromolecules [30]. However, in our study, MPL was prepared in DMSO to increase permeability [31]. Permeable molecules are often taken up by Langerhans cells, which then migrate to the draining lymph node followed by differentiation into mature dendritic cells (DC), which will stimulate neighbouring T lymphocytes [32]. These skin DC and keratinocytes play an important role in activation of immune responses after gene gun immunisation [33,34]. Although MPL was administered with DMSO to aid penetration of the skin, topical application alone might not have been the best way to administer this adjuvant. Hence, the adjuvant was further co-administered with the pLamp vaccine via intramuscular route.

In trial 1, evidence that pLamp DNA vaccine construct containing both $CD4^+$ and $CD8^+$ CTL epitopes was efficient in inducing immune responses characterised by proliferation of memory $CD4^+$ and $CD8^+$ T cells and their production of IFN-γ has been presented. This confirmed that *E. ruminantium* peptides were expressed *in vivo* after immunisation resulting in successful T cell activation. Immune responses induced *in vivo* corresponded with the responses induced *in vitro* by pLamp following electroporation of immune PBMC. These immune responses were further improved in trial 2 by co-administration of pLamp with MPL via intramuscular inoculation leading to protection of three of the five sheep against laboratory tick infection. The improved immune responses coincided with significantly improved protective efficiency of pLamp.

This highlights the importance of including adjuvants to vaccine formulations owing to the fact that they activate TLRs that drive the maturation of DC and APC, activates naïve T cells, and increase immunogenicity [35,36]. MPL has been very effective as an adjuvant and studies have shown that co-administration of this

adjuvant with the vaccine antigen is vital for induction of a successful immune response during transcutaneous immunisation [37]. When investigating the adjuvant activity of AS04, MPL adsorbed to aluminium salt, it was shown that the adjuvant and the vaccine antigen should be injected at the same intramuscular site in order to elicit effective adjuvant activity and this should be done within 24 hours [38]. The improved protective efficiency of pLamp by co-administration with MPL highlights the importance of inducing innate immunity for successful vaccination against heartwater.

When evaluating cellular immune responses induced in the immunised sheep, there was evidence of lack of correlation between these responses, their strength and protection. This represents one of the greatest challenges for pathogens like *E. ruminantium*. In this study, there was evidence of IFN-γ production in animals that did not survive challenge. A challenge study in goats showed that IFN-γ could be used as an indicator for protective immunity [39]. However, this was not the case in our study; some of the sheep that survived challenge did not show any significant IFN-γ production although one did. Previous heartwater challenge studies have also observed a lack of correlation between IFN-γ production and protective immunity [25,26,40] highlighting a need to identify additional correlates of protective immunity.

In addition to IFN-γ responses, memory T cell responses were analysed and these responses were highly variable and also showed evidence of lack of correlation with protection as observed with the IFN-γ responses. Although the memory T cell responses were variable amongst the individual sheep, there was evidence that these responses were enhanced when pLamp was co-administered with adjuvant. This correlates with other studies where co-administration of *M. tuberculosis* and *Plasmodium yoelli* subunit vaccines with MPL via intramuscular inoculation could enhance effector memory $CD4^+$ and $CD8^+$ T cells [41,42]. The enhanced memory T cell responses we observed correlated with improved protection showing the importance of these responses in successful vaccination to *E. ruminantium* infection.

Furthermore, in our animal trials we used outbred natural hosts, which possess MHC class I and II genes that are highly polymorphic. Polymorphism amongst the different alleles can affect their peptide binding abilities [43]. Different MHC class II alleles were observed amongst the different sheep with some being shared between the animals. The sheep that survived challenge had allele set *0702/*0703; allele *0203 as well as two unknown alleles that did not correspond with any of the published alleles. Although ovine MHC class II DRB1 alleles can affect immune responses to infection [44], in our case no clear correlation between MHC class II alleles and protection was observed. Interestingly none of the surviving sheep shared any alleles with the sheep that were used for the selection of epitopes *in vitro* showing that our epitopes are capable of binding to several different alleles and therefore stand a better chance of protecting a genetically diverse population. To improve our understanding of protective immunity to heartwater, transcriptome sequencing should be performed. This will aid in elucidating in depth, which immunological pathways contributed to the protection against the disease and give an indication to correlates of protection.

Previous vaccine trials showed that the DNA vaccine should be administered via both intramuscular and intradermal route to be effective [24]. In the current study the same administration method was followed and the protective efficacy was further improved by co-administration of the adjuvant intramuscularly. Whether, the vaccine will be effective following intradermal or intramuscular injection alone is not known and will be investigated in future. To further improve this vaccine, the use of alternative delivery methods that enhance DNA expression like electroporation [45,46], vector systems that increase immunity such as Lumpy skin diseases virus vector [47] and modified Vaccinia Ankara virus vector [48], and that can allow for effective administration and protection following single dose administration like nanoparticles [49], will be investigated.

In conclusion, we have presented evidence to show that the protective efficiency of pLamp multi-epitope DNA vaccine, which contains epitopes that induce a wide range of Th1 cellular immune responses, could be significantly enhanced by co-administration with MPL adjuvant via intramuscular inoculation. The importance

of including appropriate adjuvants as well as the route of administration was highlighted in this study and this will aid in future vaccine development studies. A better understanding of how some animals in a population are better protected than others could be crucial to developing a higher efficacy vaccine.

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