SUPPLEMENTARY DATA

1. Supplementary Methods 1

1.1. Infection and challenge of sheep as a source of immune PBMC

Three merino sheep (6821, 6822 and 6823) were infected and challenged as described previously [16]. Briefly, uninfected *A. hebraeum* nymphs were fed on a sheep that had been infected intravenously with *E. ruminantium* (Welgevonden strain). The engorged nymphs were allowed to moult to adult ticks in the acaridarium. The infectivity of a percentage of the adult ticks was determined using the pCS20 qPCR. Sheep 6821, 6822 and 6823 were infected with *E. ruminantium* (Welgevonden strain) by feeding infected adult ticks on them. The sheep were then monitored for heartwater symptoms and treated with Terramycin®100 (Pfizer) upon febrile reaction. Sheep were challenged 30 days later using *E. ruminantium* (Welgevonden strain) infected adult ticks to ensure that they are immune to heartwater. These sheep served as the source of immune PBMC that were used to test immune responses induced by the peptide pools *in vitro*.

1.2. IFN-γ ELISpot assay

Heartwater immune PBMC were obtained from the infected and challenged sheep 6821, 6822 and 6823 [16]. The PBMC were stimulated with a cocktail of peptides in pool 1 and pool 2 (50 µg/ml), *E. ruminantium* crude antigen (1 µg/ml, positive control) and Concanavalin A (ConA, 5 µg/ml, positive control). The unstimulated PBMC with medium only served as a negative control. The number of IFN-γ producing cells was determined using the Bovine IFN-γ ELISpotPLUS kit (Mabtech) according to the manufacturer's instructions. Briefly, PBMC (2×10^5 PBMC/well) and stimulants were seeded in pre-coated plates and incubated for 48 h at 37°C in a humidified 5% CO_2 incubator. Plates were developed and spot forming cells (SFC) were enumerated using the Zeiss KS ELISPOT Reader. Only peptide pools that had at least 10 or more spmc and had significant p values ($p \leq 0.05$ as determined by Student's t-test) were regarded as positive.

1.3. Cytokine profiling - quantitative real-time PCR (qPCR)

The cytokine profile in response to the peptides in pool 1 and pool 2 was measured by qPCR as previously described [4;5]. Briefly, immune PBMC (8 x 10^6 cells) obtained from heartwater immune sheep 6821 and 6823 [16] were stimulated with peptides in pool 1 and pool 2 (total peptide concentration 50 µg/ml) or incubated with medium only (negative control) in duplicate for 18h at 37°C, in a humidified 5% CO_2 incubator. Eighteen hours post incubation, total RNA was isolated from the stimulated PBMC using TRI REAGENT™ (Sigma) and contaminating genomic DNA was removed from the isolated RNA using the DNA-free kit (Ambion) according to the manufacturer's instructions. The qPCR was performed using the LightCycler® FastStart DNA MasterPLUS SYBR Green 1 kit according to the instruction manual (Roche). The expression levels of the following ovine cytokines was measured: IL-1α, GM-CSF, TNF-α, iNOS, IL-10. Cytokine gene expression was determined according to the 2-ΔΔCt method and referred to as fold increase (FI). Gene expression was reported as the normalised cycle threshold (ΔCt = Ct target cytokine gene − Ct housekeeping gene and ΔΔCt = ΔCt stimulated – ΔCt non stimulated medium only). Delta Ct values were calculated as the mean of duplicate samples each normalised to its own reference genes (GAPDH and β actin). The cytokines were considered to be upregulated if the mRNA increase difference was FI>1 compared to both housekeeping genes.

2. Supplementary Methods 2

2.1. Cell surface staining

Following electroporation and incubation, the cells were stained using the following commercial monoclonal antibodies: CD4 (IgM, cell line GC50A); CD8 (IgG1, cell line CACT80C) and CD45RO (IgG3, cell line ILA116A) (Washington State University Monoclonal Antibody Center, Pullman, WA) at a 1:100 dilution in stain buffer (1 x PBS containing 0.5% FBS and 0.2% sodium azide). Cells were then washed twice in stain buffer and incubated for 15 min in the dark with the corresponding secondary antibodies, which included goat anti-mouse IgM-Allophycocyanin (AF) (Invitrogen), goat anti-mouse IgG1-PE and goat anti-mouse IgG3-FITC (Serotec) at a dilution of 1:10, 1:40 and 1:10 respectively. Immunoglobulin isotype controls (MCA692, IgM, MCA928, IgG1, MCA2063, IgG3 (AbD, Serotec, Biorad)) were also included. Cells were then washed as described above and fixed with 0.2% formaldehyde in PBS. The cells were analysed using the FC 500 flow cytometer (Beckman Coulter) and data was analysed using Kaluza version 1.2 software (Beckman Coulter). Values ≥ 1% and two times higher than negative control were considered as positive.

2.2. Intracellular cytokine staining

Intracellular cytokine staining was done using the BD Cytofix/Cytoperm™ Kit (BD Biosciences) following the instructions of the manufacturer. Briefly, 4h prior to harvesting the electroporated cells, Golgi stop solution was added to the cells. The cells were then surface stained as described above and subsequently, intracellular IFN-γ staining was performed with fluorochrome-conjugated anti-cytokine antibody (Alexa fluor®488, Serotec) at a dilution of 1:20. The cells were incubated at 4°C for 30 min in the dark, followed by washing with the supplied buffers. The cells were analysed on a FC 500 flow cytometer (Beckman Coulter) and data analysed using Kaluza version 1.2 software (Beckman Coulter). Values ≥ 1% and two fold higher than negative control were considered as positive.

3. Supplementary Methods 3

3.1. Precipitation of pDNA onto gold particles

Twenty five mg gold particles of 1.6 µm diameter (BioRad) were suspended in 250 µl 0.05 M spermidine (Sigma). Thereafter, 250 µg of DNA was added to the gold/spermidine solution followed by precipitation on to the gold beads by adding 250 µl of 1 M $CaCl_2$ drop wise with continuous vortexing. The mixture was incubated at room temperature for 10 min, followed by three washes with 100% ethanol, and finally resuspended in 0.1 mg polyvinylpyrollidone (PVP) (BioRad) at a final volume of 3 ml.

3.2. Preparation of gold-coat tubing

Before coating, the Tefzel tubing (BioRad) was dried in the gene gun tubing prep station (BioRad) with nitrogen gas (0.4 psi) for a minimum of 20 min. The pDNA coated gold particle solution was introduced into the dry polypropylene tubing and placed in the tubing prep station (BioRad) leaving the gold to settle for 3-5 min. The ethanol/PVP was removed, and the tubing continuously rotated to spread the gold/pDNA uniformly. Lastly, the pDNA/gold in the tube was dried with nitrogen flow for 5-10 min. The tubing was removed and cartridges were cut using a tubing-cutter (BioRad). One preparation contained approximately 50 cartridges, which were kept dry in a cartridge storage vial with desiccant pellets. One cartridge contained 5 µg of pDNA precipitated on 0.5 mg gold particles.

4. Supplementary Tables

Supplementary Table 1. Amino acid sequences of $CD8^+$ CTL and $CD4^+$epitopes and Th1 cytokines induce *in vitro* by individual peptides. In bold are the peptides selected for construction of pSignal plus and pLamp multi-epitope DNA vaccines.

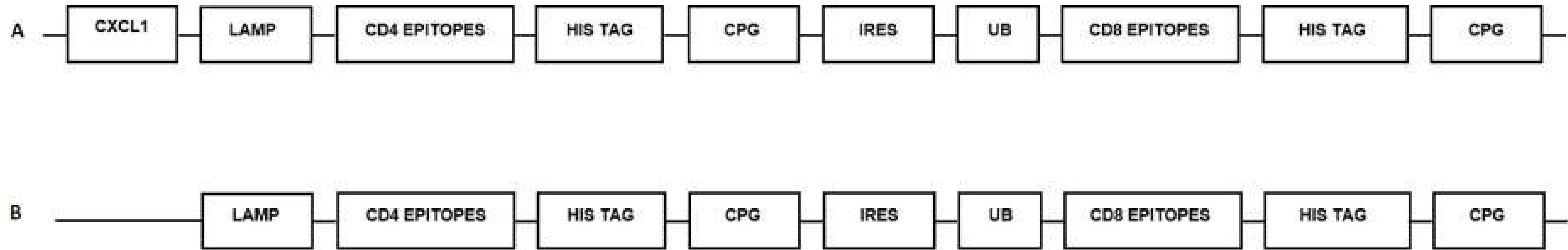
$CD8^+$ CTL epitopes		
Peptide ID	aa sequence	**Cytokines induced *in vitro* by the individual peptide [2,3]**
p2540-6	KNRTINIGVEFRIQDG	IFN-γ
p2540-16	HDNLNTKELSISLRIK	IFN-γ
p2540-19	IPQEKVIILNRFLQDY	IFN-γ
p2540-20	LNRFLQDYVNQENLGL	IFN-γ
$CD4^+$ epitopes		
p2540-21	VNQENLGLINFWKKK	IFN-γ
p7140-6	ELDKVELPKTRARETS	IFN-γ, IL-1α
p7140-7	KTRARETSSDITVISD	IFN-γ, IL-1α
p7140-12	QQCCECSSSFGVKWSH	IFN-γ, IL-1α
p7140-13	SFGVKWSHNDTSDKSD	IFN-γ, IL-1α
p7140-20	KAEDKVVKAAQIQDVP	IFN-γ, IL-1α
p7320-8	VYNITRVSSTSSSSLS	IFN-γ, GM-CSF
p7320-9	STSSSSLSTLSPTTII	IFN-γ, GM-CSF
p7320-21	IVSSDTSNNGSVAEEN	IFN-γ, GM-CSF
p7350-9	MVSICCQGTSLGGFSE	IFN-γ
p7620-2	HPKKDSLIFVQDGFSL	IFN-γ, TNF-α
p7620-12	TKLKRMGYKIYNVIFA	IFN-γ, TNF-α
p8010-8	LGSSIMAIFGKLPWPA	IFN-γ, iNos

Supplementary Table 2. Summary of sheep treatment in the vaccine trials.

Experiment	Treatment	Number of sheep/group	Dose and route of administration
Animal trial 1			
	Empty vector	5	200 µg IM; 50 µg gene gun[a]
	Empty vector + adjuvant	5	200 µg IM; 50 µg gene gun with 20 µl adjuvant topically
	pLamp + adjuvant (gene gun) and pLamp IM no adjuvant	5	200 µg IM; 50 µg gene gun with 20 µl adjuvant topically
	pSignal plus + adjuvant (gene gun) and pSignal IM no adjuvant	5	200 µg IM; 50 µg gene gun with 20 µl adjuvant topically
	Naive	2	None
	Positive control (Infection and treatment)	2	
Animal trial 2			
	Empty vector co-administered with adjuvant	2	200 µg IM co-administered with adjuvant; 50 µg gene gun with 20 µl adjuvant topically
	pLamp co-administered with adjuvant	5	200 µg IM co-administered with adjuvant; 50 µg gene gun with 20 µl adjuvant topically
	Naive	2	None

[a] gene gun = ID immunisation using a gene gun

5. Supplementary Figures



Supplementary Figure 1. Schematic representation of the two constructs used in the study. (A) represents pSignal plus and (B) represents pLamp. CD4 epitope sequences had GPGPG spacers between them while CD8 epitope sequences had AYY spacers.