Identification and characterisation of conserved epitopes of *E. ruminantium* that activate Th1 CD4$^+$ T cells: towards the development of a multi-epitope vaccine

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Highlights:

- Recombinant proteins induced IFN-γ production and other Th1 cytokines
- Peptides induced IFN-γ production and activation of memory CD4$^+$ T cells
- Promising candidates for a multi-epitope vaccine against *E. ruminantium*

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Abstract

Several studies have shown that cytotoxic T lymphocytes (CTL) require CD4+ Th1 epitopes to generate strong immune responses to intracellular pathogens. However, not much is known about *Ehrlichia ruminantium* epitopes, particularly those that can be considered potential candidates for inclusion in a multi-epitope vaccine. In order to identify $CD4^+$ Th1 epitopes that induce IFN-γ, a number of proteins previously identified as immunogenic were first screened to determine if they induce cellular immunity in tick infected immune sheep PBMC. Significant IFN-γ production and other Th1 cytokines were evident for 10 recombinant proteins in all sheep tested. Secondly, peptides (n=246) derived from the top 10 *E. ruminantium* vaccine candidate proteins were assayed using enzyme linked immunospot (ELISPOT) assay, quantitative real-time PCR and flow cytometry. Of the 246 peptides, 23 peptides, Erum0660 (p0660-42), Erum1150 (p1150-18, p1150-19), Erum2540 (p2540-6, p2540-16, p2540-19, p2540-20, p2540-21), Erum5420 (p5420-13, p5420-14), Erum7140 (p7140-6, p7140-7, p7140-12, p7140-13, p7140-20), Erum7320 (p7320-8, p7320-9, p7320-21), Erum7350 (p7350-9), Erum7360 (p7360-8), Erum7620 (p7620-2, p7620-12) and Erum8010 (p8010-8) were identified that stimulate the best and different cell mediated immune responses. Amino acid sequences of these peptides except for p7140-12, p7140-13, p7140-20, and p7350-9 were conserved between 13 different local strains. These peptides could efficiently induce memory $CD4^+$ T cells to rapidly proliferate and significantly increase IFN-γ production in immune sheep PBMC. The upregulation of pro-inflammatory cytokines, which include, IL-1α, IL-2, IL-12p40, TNF-α, IFN-γ, inducible nitric oxide synthase (iNOS) and granulocyte-macrophage colony stimulating factor (GM-CSF) was also detected. Our results show that these peptides could serve as promising candidates for a multi-epitope vaccine against *E. ruminantium*.

Keywords: *E. ruminantium*, antigens, IFN-γ, $CD4^+$ T cells, peptides

Abbreviations: cytotoxic T lymphocytes (CTL), inducible nitric oxide synthase (iNOS), granulocyte-macrophage colony stimulating factor (GM-CSF)

1. INTRODUCTION

Heartwater, a tick borne disease of domestic ruminants, constitutes a major problem to the economy and introduction of more productive breeds in endemic areas in sub-Sahara Africa. It is caused by a parasitic rickettsia *Ehrlichia ruminantium* (Moshkovski, 1947; Dumler et al., 2001). The only commercially available vaccine is a live blood vaccine with numerous drawbacks of which the most important being that it does not adequately protect against all virulent genotypes (Allsopp, 2009). Development of an effective and improved subunit vaccine would offer tremendous ethical, economic and social benefits. In general, approaches used to identify vaccine candidates for recombinant vaccine development are guided by the type of immune responses that are likely to mediate protection.

Cell mediated immune responses (CMI) has been shown to play a key role in protection against heartwater (reviewed by Totté et al., 1999a). The protective antigens of *E. ruminantium* are still not precisely defined, but several studies have indicated that immunisation of animals with *E. ruminantium* induces immune responses that are characterised by the production of IFN-γ and T cell proliferative responses. For example, purified proteins of *E. ruminantium* were shown *in vitro* to stimulate lymphocytes to proliferate and produce IFN-γ from infected cattle, sheep and goats inoculated with inactivated organisms (Totté et al., 1999b; van Kleef et al., 2000; 2002; Esteves et al., 2004). Similarly, when mice were primed by a DNA vaccine and then inoculated with recombinant proteins of *E. ruminantium*, immune responses were characterised by production of IFN-γ and IL-2 (Simbi et al., 2006). The most promising vaccination results were obtained when a cocktail of four *E. ruminantium* open reading frames (1H12) was tested in sheep either as a DNA vaccine alone or using a DNA prime/recombinant protein boost vaccine (Collins et al., 2003a; 2003b; Pretorius et al., 2007; 2008). Both regiments elicited complete protection against needle challenge. Reverse vaccinology techniques have identified additional *E. ruminantium* (Welgevonden) vaccine candidate genes from the annotated genome sequence (Collins et al., 2005; Sebatjane et al., 2010; Liebenberg et al., 2012). These included *E. ruminantium* proteins with unknown function, membrane-associated proteins and proteins containing tetratricopeptide or ankyrin repeat domains or tandem repeats (Liebenberg et al., 2012). The latter three repeat members have been implicated in host–pathogen interactions (Jernigan and Bordenstein, 2015; Sharma and Pandey, 2016). Furthermore, surface associated or secreted proteins have been shown to be protective antigens against several pathogens including *Chlamydia* (Faludi and Szabó, 2011; Koroleva et al., 2017), *Ehrlichia canis* and *Ehrlichia chaffeensis* (Doyle et al. 2006; Nandi et al., 2007), *Mycobacterium tuberculosis* (Villarreal et al., 2014) and *Anaplasma marginale* (Morse et al., 2012a, b).

The protective abilities of the reverse vaccinology selected ORFs, which induced cellular immune responses *in vitro*, were then tested *in vivo*. However, at most 20% survival was obtained after needle challenge in sheep immunised with DNA constructs consisting of these ORFs (Sebatjane et al., 2010). In the 1H12 study, it has been shown that animals that gave 100% protection against an *E. ruminantium* needle challenge were not immune to a heartwater-infective tick field challenge (Collins et al., 2003a; 2003b; Pretorius et al., 2007; 2008). Thus, there is evidence to suggest that a needle challenge model does not mimic natural infection. This may be explained by differential gene expression studies in bacteria derived from the host in comparison with those derived from ticks. It has been shown previously that *A. phagocytophilum* genes expressed by infected sheep were differentially expressed in animals experimentally infected as compared to naturally field-infected animals and the genes were either up-regulated in experimentally infected sheep or down-regulated in naturally tick infected animals (Galindo et al., 2008). In *E. ruminantium*, similar differential gene expression was observed between mammalian and tick host cells (Tjale et al., 2018). Therefore, in the present study, we attempted to mimic natural tick infection more closely by first screening all the previously selected *E. ruminantium* vaccine candidates for their ability to induce CMI in PBMC isolated from sheep experimentally infected with heartwater by ticks.

One complicating factor to consider is that whole proteins from pathogens contain epitopes, which may inhibit protective immune responses or induce immunopathology (Wang et al., 2005; Gowththaman and Agrewala, 2008). Excluding these inhibitory epitopes and including only the protective epitopes from multiple antigens will allow efficient use of limited space needed to package numerous antigens in a recombinant vaccine. The identification and characterisation of vaccine candidate peptides derived from the proteins of different pathogens has been studied extensively for example: *Neospora caninum* (Staska et al., 2005), *Brugia malayi* (Madhumathi et al., 2010), *Mycobacterium tuberculosis* (Nair et al., 2014) and *Plasmodium falciparum* (Sedegah et al., 2010). As such, research directed at elucidating the epitopes of selected *E. ruminantium* recombinant proteins will provide a better understanding of which fragment of the protein is immunogenic for incorporation into a multivalent vaccine.

Hence, in this study, several assays were used to identify vaccine candidate proteins and epitopes. These included IFN-γ ELISPOT, reverse transcriptase- quantitative real-time PCR (RT-qPCR) amplification of cytokines and flow cytometry. Ten of the recombinant proteins induced CMI in PBMC thus demonstrate that they were recognised by PBMC from *E. ruminantium* tick infected animals. Similarly, their epitopes activating $CD4^+$ T cells were determined. This resulted in the selection of 23 vaccine candidate peptides that could efficiently induce memory $CD4^+$ T cells to rapidly proliferate and significantly increase IFN-γ

production in immune sheep PBMC. These peptides will be investigated in future for inclusion in a multi-epitope vaccine that can induce a wide array of cytokines.

2. MATERIALS AND METHODS

2.1 Expression of *E. ruminantium* proteins

Protein expression was performed using the pET102/TOPO® expression system (Invitrogen) according to the instructions of the manufacturer, as described previously (Liebenberg et al., 2012). Briefly, 27 *E. ruminantium* ORFs listed in Table 1 were PCR amplified using specifically designed primers and the amplicons were cloned in the pET102/TOPO® expression vector. Plasmids containing the expected insert size were sequenced to confirm the correct sequence and that the ORFs were in-frame. Recombinant (His_6-tagged) proteins were purified from supernatants or inclusion bodies using the Protino® Ni 150 prepacked columns kit (Macherey-Nagel) according to the instructions of the manufacturer. The proteins were purified and acetone precipitated. The concentration of the recombinant proteins was determined using the RC DC Protein Assay (Biorad). They were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using anti-His_6 antibodies (Roche) and used in the immune assays listed below to select proteins used for peptide synthesis.

2.2 Synthesis of peptides

A total of 246 overlapping peptides (16-mer, each overlapping by 8 amino acids) spanning the length of 10 proteins were synthesised by Genscript (USA). The purity of the peptides was >98% as analysed by high-performance liquid chromatography. The peptides were dissolved in 100 µl 100% dimethyl sulfoxide and then diluted with PBS to 1 mg/ml and stored at -70°C. Peptides were diluted to 100 µg/ml in Roswell Park Memorial Institute (RPMI)-1640 (GIBCO® RPMI + GlutaMAXTM-I) (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS), 55 mM 2-mercaptoethanol and 1% GIBCO® Pen Strep (Invitrogen) (complete RPMI) prior to use in immunological assays.

2.3. Animals and Ethical statement

Merino sheep (n=4) 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 (Steyn et al., 2008). 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 (DAFF) under section 20 of the Animal Diseases Act of 1984 with reference (12/11/1/1).

2.3.1 Inoculation of animals as a source of immune mononuclear cells

To mimic field/natural immunisation, three sheep (s6821, s6822, s6823) were treated with ticks infected with the Welgevonden strain in the laboratory as described previously (Thema et al., 2016). Briefly, uninfected *A. hebraeum* nymph ticks were infected by feeding on a sheep that had been infected intravenously with *E. ruminantium* Welgevonden stock. Engorged nymphs were allowed to moult to adults in the laboratory. The sheep were then infected by feeding 10 adult (5 males and 5 females) heartwater infected ticks on it. The sheep were monitored daily for the onset of clinical signs and treated on the third day of febrile reaction with Terramycin®100 (Pfizer). The sheep were tick challenged 30 days after primary infection with the Welgevonden infected ticks. Heartwater infection of ticks and sheep were confirmed by pCS20 real-time PCR (Steyn et al., 2008).

2.4 Immunological assays

2.4.1. Purification of PBMC

PBMC were purified from sheep blood under sterile conditions as described previously (Liebenberg et al., 2012). Briefly, blood from a sheep was collected from the jugular vein in BD Vacutainer® EDTA tubes (Becton, Dickinson) and PBMC were isolated by density gradient centrifugation (Histopaque®-1077; Sigma–Aldrich®). The cells were washed three times and counted using a TC10™ Automated cell counter (BioRad) and the cells resuspended (4×10^6 cells/ml) in complete RPMI.

2.4.2 IFN-γ ELISPOT assay

The bovine/ovine/equine IFN-γ ELISPOT PLUS kit (Mabtech) was used according to the manufacturer's instructions. PBMC (2×10^5 cells/well) were seeded in triplicate in pre-coated ELISPOT 96 well plates. PBMC were stimulated with *E. ruminantium* crude antigen (1 µg/ml, positive control), Concanavalin A (ConA, 5 µg/ml, positive control), *E. ruminantium* recombinant proteins (10 µg/ml) or synthetic peptides (50 µg/ml*)*. Negative controls included were Erum5400 and its peptide, p5400-2+3 (internal negative control), or medium only (unstimulated PBMC). Peptides were either tested individually or as pools depending on the amount of PBMC isolated at a specific time point. The plates were developed after 48 h incubation at 37°C in a humidified 5% CO_2 incubator. The number of spots per million cells (spmc) in the antigen stimulated wells was compared to the number of spmc of the

corresponding negative control. Only the number of spmc that were two times higher than the negative control and with a significant *P*-value ($p \leq 0.05$) as determined by Student's t-test were regarded as positive. Additionally, for peptide responses only samples with at least 10 spmc were considered positive.

2.4.3 Cytokine profiling using RT-qPCR

RT-qPCR was performed as described previously (Liebenberg et al., 2012). The RT-qPCR primers used to quantitate the cytokine levels were previously optimised for ovine: IL-1α, GM-CSF and transforming growth factor beta (TGF-β) (Smeed et al., 2007), IFN-γ, IL-12, glyceralderhyde-3-phoshate dehydrogenase (GAPDH), and TNF-α (Bhudia et al., 2006), iNOS (Wood et al., 2005), IL-10 and IL-18 (Markus et al., 2007), IL-2 (Umeshappa et al., 2010), β-actin and TLR4 (Kabaroff et al., 2006). Total RNA was isolated from PBMC (8×10^6 cells) which were stimulated with recombinant proteins and peptides added at a final concentration of 50 µg/ml for 18 h in duplicate at 37°C, in a humidified 5% CO_2 incubator. Negative controls included were Erum5400 and its peptide, p5400-2+3 (internal negative control), or medium only (unstimulated PBMC). The RNA was isolated using TRI REAGENT™ (Sigma) according to the instructions of the manufacturer. Contaminating genomic DNA was removed from the isolated RNA by using the DNA-free kit (Ambion) according to the manufacturer's instructions. The RT-qPCR was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 kit according to the instruction manual (Roche). The RT-qPCR cycling conditions for all the cytokines were: 10 min at 94°C; 50 cycles of 15 s at 94°C, 30 s at 58°C and 20 s at 72°C; followed by melting starting at 65°C rising to 95°C at 0.3°C per second; and cooling to 40°C. Cytokine gene expression was determined according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) 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, common housekeeping genes, were used to normalise cytokine gene expression. One FI indicates that the cytokine mRNA concentration of the stimulated sample is twice that of non-stimulated samples, and the cytokines were considered to be upregulated if the mRNA increase difference was FI > 1 compared to both housekeeping genes.

2.4.4 Cell surface staining

Immune PBMC (2×10^5 cells/well) were seeded in triplicate and stimulated with antigens for 72 h at 37°C, in a humidified 5% CO_2 incubator. Negative controls included were Erum5400 and its peptide, p5400-2+3 (internal negative control), or medium only (unstimulated PBMC).

The cells were stained with the following commercial monoclonal antibodies: CD4 (IgM, cell line GC50A) and CD45RO (IgG3, cell line ILA116A) (Washington State University Monoclonal Antibody Center, Pullman, WA) at a 1:100 dilution in PN buffer (1 x PBS containing 0.5% FBS and 0.2% sodium azide). Following washing, the corresponding secondary antibodies: goat anti-mouse IgM-Allophycocyanin (APC) (Invitrogen) and goat anti-mouse IgG3-FITC (Serotec) were added at a dilution of 1:10. Immunoglobulin isotype controls (MCA692, IgM, MCA2063, IgG3 (AbD, Serotec, Biorad)) were included in order to test nonspecific binding of the secondary antibodies. All incubations were for 15 min at room temperature and washing was done twice with PN buffer. Cells were fixed with 0.2% formaldehyde in PBS. Samples were assayed on a FC 500 Beckman Coulter flow cytometer and data analysed using Kaluza version 1.2 (Beckman Coulter). Values ≥ 1% and two times higher than negative control were considered as positive.

2.4.5 Intracellular IFN-γ staining

Intracellular IFN-γ staining using the BD Cytofix/Cytoperm™ Kit (BD Biosciences) and protocol was done. Briefly, PBMC (2 x 10^5 cells/well) were seeded in triplicate and incubated for 72 h at 37°C in the presence or absence of 50 µg/ml proteins/peptides. Golgi stop solution was added 4 h prior to harvesting. Cells were first surface stained as described above and subsequently, intracellular IFN-γ staining was performed with fluorochrome-conjugated anti-cytokine antibody (Alexa fluor®488, Serotec) and an isotype control, IgG1 (AbD, Serotec, Biorad) 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 with an FC 500 Beckman Coulter flow cytometer and data analysed using Kaluza version 1.2 (Beckman Coulter). Values ≥ 1% and two fold higher than negative control were considered as positive.

2.5. Statistical analysis

The significance of differences between immunological assay results was determined by means of the Student's *t*-test. Differences with *P*-value ($p \leq 0.05$) were considered significant. Significance was calculated by comparing biological repeats (3 sheep) of stimulated PBMC to the negative peptide control.

3. RESULTS

3.1 Screening of recombinant proteins using PBMC of *E. ruminantium* immune sheep after tick infection and challenge

Twenty seven recombinant proteins were selected from proteins previously tested as vaccine candidates or that were able to induce cellular immunity in our laboratory (Table 1). These

were previously cloned in pET102 TOPO vectors: Erum2540 Erum2550, Erum2580 Erum2590 (Pretorius et al., 2008); Erum7340, Erum7350, Erum7360 and Erum7380 (Sebatjane et al., 2010); Erum0660, Erum1050, Erum1150, Erum1990, Erum2330, Erum4530, Erum5400, Erum5420, Erum7140, Erum7280, Erum7320, Erum7620 and Erum8010 (Liebenberg et al., 2012); and Erum5000, Erum7760, Erum8060 (Thema et al., 2016), Erum0590, Erum2300 and Erum3750 (unpublished). For the present study, the proteins were re-expressed in *E. coli* and the presence of a his-tagged protein of correct size was shown by western blot (data not shown) prior to use and all were expressed as previously reported (Table 1). Thereafter, all were included in all assays as positive controls for peptide evaluation.

The proteins were then screened to show that they were recognised by PBMC from tick infected sheep, as previously found in PBMC from sheep challenged by needle administration of *E. ruminantium*. The ability of *E. ruminantium* proteins to induce IFN-γ production *in vitro* was evaluated by the ELISPOT assay and RT-qPCR using PBMC obtained from three tick challenged sheep, s6821, s6822 and s6823. All three sheep developed a febrile response after primary infection and were treated but none developed heartwater symptoms after challenge, indicating that they were immune. PBMC from all sheep tested positive when stimulated with the crude Welgevonden antigen showing *E. ruminantium* specific recall responses. As illustrated in Table 2, all proteins induced significant IFN-γ production in at least one sheep as compared to unstimulated PBMC and selected internal negative recombinant protein ($p \leq 0.05$). Other cytokines induced by the presence of these proteins were also evaluated. Each recombinant protein engendered a different response yielding different cytokine profiles. Of these, 10 recombinant proteins were selected for peptide synthesis since each induced IFN-γ in at least two animals tested as well as at least one of a particular cytokine within the top three highest FI levels (Table 2). These were Erum0660 (IL-18; TNF-α), Erum1150 (IL-2; iNOS, TLR4), Erum2540 (IFN-γ), Erum5420 (IL-12, TGF-β), Erum7140 (IL-1α; IL-18), Erum7320 (GM-CSF), Erum7350 (IFN-γ), Erum7360 (IL-2; IL-12; IL-18; TLR4; TGF-β), Erum7620 (IL-1β, TNF-α, iNOS) and Erum8010 (iNOS, IFN-γ). Erum5400 was one of the proteins that did not engender a response in the tick challenged immune PBMC and was used as a negative control in all subsequent assays. These proteins gave reproducible results in PBMC from all animals tested and were further investigated to identify the epitopes that induce these responses.

Table 1. A summary of results previously obtained and the characteristics of the recombinant proteins and the ORFS encoding them tested in this study. Previously obtained results shown here are from vaccine experiments with needle challenge and immune assays done with immune PBMC obtained from sheep infected with *E. ruminantium* by needle challenge.

Recombinant proteins	Function	Length (aa)	MW (kDa)	Solubility	Yield (µg/ml)	Reference	% Protection after needle challenge	CMI induced in immune PBMC (needle)
Erum0590	Integral membrane	613	70.69	Soluble	153	Unpublished	- [b]	+ [a]
Erum0660	Unknown	3715	48	Insoluble	75	Liebenberg et al., 2012	-	+
Erum1050	Integral membrane	545	51.64	Soluble	398	Liebenberg et al., 2012	-	+
Erum1150	Unknown	179	19.5	Soluble	400	Liebenberg et al., 2012	-	+
Erum1990	Trigger factor	446	50.78	Soluble	510	Liebenberg et al., 2012	-	+
Erum2300	Membrane	370	43.61	Soluble	578	Unpublished	-	+
Erum2330	Exported	306	34.95	Soluble	432	Liebenberg et al., 2012	-	+
Erum2540	Exported	175	20.48	Soluble	1200	Pretorius et al., 2008	100	+
Erum2550	ATP-binding protein part of ABC transporters	340	38.2	Soluble	1428	Pretorius et al., 2008	100	+
Erum2580	Periplasmic solute binding protein (SBP)	287	33.10	Soluble	488	Pretorius et al., 2008	100	+
Erum2590	ATP-binding protein part of ABC transporters	242	27.72	Soluble	1599	Pretorius et al., 2008	100	+
Erum3750	Unknown	1674	179.81	Soluble	1400	Unpublished	-	+
Erum4530	Unknown	199	23.02	Soluble	144	Liebenberg et al., 2012	-	+
Erum5000	Exported protein	490	53.57	Insoluble	1542	Thema, 2008, Thema et al., 2016	-	+
Erum5400	Unknown	173	19.8	Soluble	369	Liebenberg et al., 2012	-	+
Erum5420	GTP-binding ERA	296	33.7	Soluble	423	Liebenberg et al., 2012	-	+
Erum7140	Membrane	197	21.89	Soluble	395	Liebenberg et al., 2012	-	+
Erum7280	Membrane	181	21.06	Soluble	402	Liebenberg et al., 2012	-	+
Erum7320	Integral membrane	266	28.12	Soluble	412	Liebenberg et al., 2012	-	+
Erum7340	Membrane LMW	122	13.32	Soluble	239	Sebatjane et al., 2010	20	+
Erum7350	Membrane LMW	145	16.17	Soluble	500	Sebatjane et al., 2010	20	+
Erum7360	Membrane LMW	147	16.13	Soluble	194	Sebatjane et al., 2010	20	+
Erum7380	Membrane LMW	157	17.23	Soluble	608	Sebatjane et al., 2010	20	+
Erum7620	Integral membrane	120	14.43	Soluble	395	Liebenberg et al., 2012	-	+
Erum7760	Exported lipoprotein	250	29.34	Soluble	654	Thema, 2008, Thema et al., 2016	-	+
Erum8010	Integral membrane	118	12.28	Soluble	244	Liebenberg et al., 2012	-	+
Erum8060	Exported protein	204	23.50	Soluble	1700	Thema, 2008, Thema et al., 2016	-	+

[a] Tested positive
[b] not tested *in vivo*

Table 2. Summary of immune responses induced by *E. ruminantium* recombinant proteins *in vitro* in PBMC from infected tick challenged sheep. These proteins were investigated for inclusion in a multi-epitope vaccine that can induce a wide array of cytokines. The three highest FI for a particular cytokine is indicated in bold.

Recombinant proteins	ELISPOT spmc for sheep number (SPMC ±SD; p≤0.05[a])			Major cytokines induced in tick immune PBMC in at least two of the sheep tested (RT-qPCR)	Number of peptides synthesised per ORF
	s6821	s6822	s6823	Cytokine (relative FI)	
Erum0590	**235±28**	**138±4**	**365±28**	IL-12 (15), IL-18 (16), iNOS (55), TGF-β (**27**)	nd
Erum0660	**370±74**	**358±47**	**493±67**	IL-18 (**26**), TNF-α (**96**)	52
Erum1050	**270±0**	175±28	**303±25**	iNOS (140), TLR-4 (18)	nd
Erum1150	**435±0**	**188±18**	**268±11**	IL-2 (**27**), iNOS (**179**), TLR-4 (**27**)	21
Erum1990	**260±71**	48±32	**338±39**	iNOS (87), TNF-α (**58**)	nd
Erum2300	133±95	48±39	**135±42**	iNOS (36), TNF-α (36)	nd
Erum2330	**260±57**	125±78	**358±39**	iNOS(37)	nd
Erum2540	**528±11**	**278±25**	**403±53**	IFN-γ (**322**)	21
Erum2550	0	0	**58±7**	IL-18 (4)	nd
Erum2580	**255±12**	**443±23**	**670±42**	iNOS (7), IL-18 (8), TGF-β (3), IL-1α (**5**), IL-12 (4), GM-CSF (**8**)	nd
Erum2590	0	0	**25±12**	IL-1α (**2**), IL-18 (2), GM-CSF (**3**)	nd
Erum3750	**63±4**	**90±35**	15±21	IL-12 (17), iNOS (45), TLR-4 (**19**), TGF-β (18)	nd
Erum4530	**208±4**	**110±57**	**320±106**	iNOS (32), TGF-β (20)	nd
Erum5000	**228±2**	**173±10**	**85±7**	IFN-γ (**129**)	nd
Erum5400[b]	0	0	0	IL-2 (2), TNF-α (1)	nd
Erum5420	**265±64**	**153±11**	**235±81**	IL-12 (**45**), TGF-β (**33**)	34
Erum7140	**258±67**	**120±14**	**430±14**	IL-1α (**135**), IL-18 (**21**), iNOS (132)	24
Erum7280	**40±7**	**88±39**	**448±11**	IL-12 (**19**), iNOS (59)	nd
Erum7320	**245±7**	**398±81**	**628±53**	GM-CSF (**39**), iNOS (33)	32
Erum7340	**338±39**	**73±4**	**220±28**	IL-2 (12), IL-12 (7), IL-18 (17), iNOS (48), TGF-β (5), TLR-4 (13), TNF-α (7)	nd
Erum7350	**385±99**	**238±4**	**400±71**	IFN-γ (**220**)	17
Erum7360	**353±25**	**173±25**	**203±81**	IL-2 (**231**); IL-12 (**22**), IL-18 (**56**), iNOS (29), TNF-α (17), TLR-4 (**43**), TGF-β (**70**)	17
Erum7380	**313±18**	**103±4**	**125±0**	IL-12 (7), IL-18 (15), iNOS (27), TLR-4 (12), TNF-α (4)	nd
Erum7620	**180±14**	**418±4**	**445±21**	IL-1β (**72**); TNF-α (**224**), iNOS (**170**)	14
Erum7760	**290±71**	80±57	**253±60**	IL-2 (**30**), iNOS (107)	nd
Erum8010	**713±95**	**270±7**	**433±32**	iNOS (**516**)	14
Erum8060	**335±14**	**150±14**	**273±25**	IL-2 (**19**), IL-12 (15), IL-18 (14), iNOS (92), TGF-β (13), TLR-4 (10), TNF-α (7)	nd
Ag+	**165±12**	**268±18**	**233±20**	Nd	
ConA	**303±129**	**407±25.5**	**178±27.5**	Nd	

[a]Significant increase in spots per million cells is indicated in bold (if stimulated PBMC is compared to unstimulated PBMC). Only samples with at least 10 spmc and had significant p values (p≤0.05 as determined by Student's t-test) were regarded as positive and are indicated in bold.
[b]Negative control protein
nd = not done

3.2 IFN-γ production by PBMC of immune sheep in response to overlapping synthetic peptides derived from *E. ruminantium* proteins

The next experiment was aimed at determining the epitope sequences of *E. ruminantium* proteins that can induce recall $CD4^+$ T CMI. A total of 246 overlapping peptides (16-mer, each overlapping by 8 amino acids) spanning the length of 10 selected proteins were synthesised. The amino acid sequences of the proteins from 13 South African isolates were then aligned to check whether the selected epitopes were conserved between the different isolates.

These 246 peptides were first screened with the IFN-γ ELISPOT assay to select peptides used for RT-qPCR screening. *E. ruminantium* crude antigen (Ag^+) induced IFN-γ production that was significant in PBMC from all three sheep. Because IFN-γ production was not induced by p5400-2/3 in PBMC from any of the sheep tested previously, it was chosen as a negative control for subsequent experiments. A total of 23 peptides induced positive and statistically significant ($p \leq 0.05$) IFN-γ production, when the average of the three biological repeat spmc values were compared to the negative control peptide data (Table 3). Average number of spots/million PBMC for individual sheep are shown in STable 2 (Supplementary information). Amino acid sequences of peptides selected from 10 promising vaccine candidates are listed in STable 1. All peptides were then screened for their ability to induce other Th1 cytokines by stimulating PBMC and using RT-qPCR.

3.3 Cytokine mRNA expression in PBMC stimulated with individual peptides by RT-qPCR analysis

A total of 23 peptides that were positive in the ELISPOT IFN-γ assay screening were evaluated by RT-qPCR. The levels of cytokine mRNA expression in PBMC after normalisation with GAPDH and β-actin reference genes are shown in STable 3 (Supplementary information). As indicated in Table 2, several recombinant proteins induced a different cytokine profile. Therefore, the cytokine that had the highest FI for each recombinant protein was used to identify the peptides that induced that cytokine. Recombinant proteins Erum1150 (iNOS), Erum7140 (IL-1α), Erum7320 (GM-CSF), Erum7350 (IFN-γ) and Erum7620 (TNF-α) induced a positive FI in PBMC from all three sheep and Erum0660 (TNF-α), Erum2540 (IFN-γ), Erum5420 (IL-12) Erum7360 (IL-2) and Erum8010 (iNOS) from two sheep. Similarly, the conserved peptides that induced responses in PBMC from all sheep tested were: p2540-6, -20 and -21 (IFN-γ); p7140-6, -7, -12, -13 and -20 (IL-1α); p7320-8, -9, and -21 (GM-CSF); p7350-9 (IFN-γ); p7620-2, -12 (TNF-α) and p8010 -8 (iNOS). Furthermore, conserved peptides that induced positive responses in PBMC from two sheep were: p0660-42 (TNF-α); p1150-18 and -19 (iNOS); p2540-16 and -19 (IFN-γ); p5420-13 and -14 (IL-12) and p7360-8 (IL-2). These conserved peptides were selected for further evaluation using phenotype analysis as indicated in Table 3. Non-conserved peptides were also included for further analysis if it was the only peptide from a protein that induced cytokine production, for example p7350-9 (IFN-γ), or high responders for example p7620-12. Moreover, similar results were obtained from PBMC stimulated with intact crude *E. ruminantium* as compared to stimulation with recombinant proteins and peptides. The cytokine mRNA levels varied between animals and reference genes used to normalise the samples.

3.4 Determining percentage of $CD4^+CD45RO^+$ T cells responsive to selected peptides

In an attempt to assess the phenotype of PBMC responsive to the 23 peptides selected (listed in Tables 2, 3) the percentage of $CD4^+$ T cells expressing memory markers ($CD45RO^+$) were measured by flow cytometry after 72 h stimulation with antigen *in vitro*. SFigure 1 (A and E) shows representative dot plots and histograms of the isotype controls. Background values varied from 0.08% to 0.24% (CD45Ro). SFigure 1 shows representative dot plots of the percentage increase of $CD4^+$ T cells expressing $CD45RO^+$ (quadrant T++) when PBMC were unstimulated or stimulated with peptide, Erum7320-P21 and the histograms show % of total $CD4^+$ cells (both $CD45^+$ and $CD45^-$). A total of 14 peptides induced an increase in memory $CD4^+$ T cells in at least two sheep tested except p0660-42; p1150-18; -19; p2540-6; -20 and p5420-13; -14 (Table 3). The nine peptides that induced the highest average % increase (20% and above) and significant $CD4^+CD45RO^+$ responses in the three sheep are p2540-21; p7140-6, -12, -13 -20; p7320-8, -9, -21 and p7620-2. These results also indicated that each peptide induced the activation of $CD4^+CD45RO^+$ T cells, and although the responses varied between animals.

3.5 Determination if $CD4^+$ T cells produce IFN-γ in response to the selected peptides

Intracellular IFN-γ staining assay was used to obtain the frequency of peptide specific IFN-γ producing $CD4^+$ T cells after stimulation of PBMC from three immune sheep (s6821, s6822 and s6823) with the 23 selected peptides. SFigure 2 shows representative dot plots of the percentage increase of $CD4^+$ T cells expressing IFN-γ when PBMC were unstimulated or stimulated with rErum2550. When PBMC from all sheep were stimulated with peptides, the assay revealed that all peptides induced significant IFN-γ production by $CD4^+$ T cells in each animal as compared to negative peptide control (Table 3). These peptides will be further analysed for possible use in the construction of a multi-epitope DNA vaccine (manuscript in preparation).

Table 3. The top 23 Th1 immune response inducing peptides. Cytokine production and phenotypic analysis of PBMC from immune sheep (s6821, s6822, and s6823) stimulated with peptides at a final concentration of 50 µg/ml. Values in red indicate significant responses induced by peptides in PBMC from all three sheep compared to the negative control. Average number of SPMC or % increase are shown ± the standard deviation for the biological repeats.

Peptides	ELISpot (Ave Spmc ± SD)	Major cytokine induced for least 2 sheep tested (RT-qPCR)	$CD4^+CD45RO^+$ T cells (AVE % increase)				$CD4^+IFN\text{-}\gamma^+$ T cells (AVE % increase)[a]			
		Cytokine (relative FI)	s6821	s6822	s6823	Ave±Stdev	s6821	s6822	s6823	Ave±Stdev
p0660-42[b]	102±133.4	TNF-α (8)	1±0	0	0	0	**2±0,1**	**2±0,2**	**2±0,3**	2±0,0
p1150-18[b]	59±59.0	iNOS (3)	0	1±0	4±0,2	2±2,1	**4±0,5**	**4±1,1**	**1±0**	3±1,2
p1150-19[b]	68±60.1	iNOS (3)	0	1±0	4±0,2	2±2,1	**4±0,5**	**4±1,1**	**3±0,3**	4±0,6
p2540-6[b]	95±25.7	IFN-γ (5)	0	**28±0,5**	**23±0,2**	17±14,9	**6±0,1**	**3±0,3**	**3±0,2**	4±1,7
p2540-16[b]	191±93.3	IFN-γ (1)	**24±0**	**18±0,2**	**3±0,1**	15±10,8	**10±0,3**	**3±0,1**	**3±1,2**	5±4,0
p2540-19[b]	93±41.1	IFN-γ (2)	**8±0,1**	**24±0,3**	**20±0,5**	17±8,3	**8±0,1**	**4±0,1**	**5±0,3**	6±2,1
p2540-20[b]	89±53.3	IFN-γ (3)	1,4±0,1	**25±0,2**	**15±0,4**	14±11,9	**6±0,1**	**3±0,3**	**7±0,2**	5±2,1
p2540-21[b]	117±42.9	IFN-γ (3)	**35±0,5**	**24±0,4**	**23±0,2**	27±6,7	**4±0,1**	**4±0,1**	**3±0,1**	4±0,6
p5420-13[b]	58±50.1	IL-12 (5)	0	1±0,1	2±0,2	1±1,0	**4±0,3**	**5±1,2**	**4±0,1**	4±0,6
p5420-14[b]	49±41.2	IL-12 (5)	0	1±0,1	2±0,1	1±1,0	**4±0,3**	**5±1,2**	**3±0,6**	4±1,0
p7140-6[b]	103±38.6	IL-1α (282)	**37±1,2**	**14±0,5**	**36±0,1**	29±13,0	**20±1,4**	**5±1,2**	**3±1,0**	9±9,3
p7140-7[b]	145±97.3	IL-1α (1789)	**49±0,7**	**6±0,5**	**22±0,1**	26±21,7	**20±1,5**	**6±1**	**9±0,1**	12±7,4
p7140-12	161±118.4	IL-1α (9)	**28±0,4**	**19±0,1**	**23±0,2**	23±4,5	**14±1,1**	**3±0,2**	**2±0,1**	6±6,7
p7140-13	99±47.1	IL-1α (487)	**33±0,7**	**16±0,3**	**23±0,3**	24±8,5	**15±1,2**	**3±0,5**	**5±0,1**	8±6,4
p7140-20	158±85.2	IL-1α (12)	**34±0,6**	**23±0,4**	**23±0,3**	27±6,4	**11±1**	**5±1,2**	**6±0,3**	7±3,2
p7320-8[b]	162±87.5	GM-CSF (2272)	**20±0,5**	**19±0,2**	**33±0,3**	24±7,8	**15±0,2**	**3±0,1**	**2±0,3**	7±7,2
p7320-9[b]	157±76.4	GM-CSF (605)	**26±0,7**	**8±0,4**	**28±0,3**	21±11,0	**6±1,1**	**3±0**	**5±0,1**	5±1,5
p7320-21[b]	121±77.8	GM-CSF (7886)	**26±0,3**	**30±0,4**	**10±0,5**	22±10,6	**6±0,5**	**3±0,5**	**3±0,5**	4±1,7
p7350-9	213±41.4	IFN-γ (2)	**11±0,5**	**26±0,4**	4±0,2	14±11,2	**7±0,3**	**3±0,3**	**3±0,2**	4±2,3
p7360-8[b]	292±82.4	IL-2 (6)	0	1±0,1	5±0,2	2±2,7	**4±0,8**	**4±1,1**	**3±1,1**	4±0,6
p7620-2[b]	210±136.4	TNF-α (1013)	**29±1,2**	**26±0,1**	**8±0,1**	21±11,4	**10±0,5**	**2±0**	**5±1,2**	6±4,0
p7620-12[b]	132±16.4	TNF-α (55)	**15±1,8**	**8±0,2**	**8±0,4**	10±4,0	**16±2,7**	**7±1,1**	**4±1,0**	9±6,2
p8010-8[b]	149±89.0	iNOS (87)	**14±1,2**	**20±0,1**	**7±0,1**	14±6,5	**15±2,4**	**4±1**	**8±0,5**	9±5,6
NC	0	0	0	0	3±1,3	1±1,7	0,5±0	0	0,2±0	0
Ag+	**531±71.6**	All(+)	**6±1,2**	**13±0,58**	**24±0,5**	14±9,1	**2±0,4**	**2±0,3**	**2±0,1**	2±0,00

[a]Significant percentage increase of memory $CD4^+$ T cells and IFN-γ producing $CD4^+$ T cells are indicated in bold. Only samples with significant p values ($p \leq 0.05$ as determined by Student's t-test) were regarded as positive. The values are expressed as means ±SD.
[b]Amino acid sequence of peptide is conserved between 13 different local isolates. NC = Negative control peptide.

4. DISCUSSION

It is well established that T cells recognise peptide epitopes presented in the context of MHC molecules, however the identification and validation of T cell epitopes is still new and a challenge for a pathogen like *E. ruminantium*. In this study, peptides derived from *E. ruminantium* proteins were identified that induced Th1 $CD4^+$ T cells to produce IFN-γ and other pro-inflammatory cytokines essential for bacterial clearance. The study provided a rationale to further identify other peptides from different *E. ruminantium* antigens as components for inclusion in a multi-epitope DNA vaccine against heartwater. Evidence for a possible role of $CD4^+$ Th1 T cell antigens in immunity to other bacterial pathogens, *Anaplasma marginale* (Brown et al., 2001; Abbott et al., 2004); *Staphylococcus aureus* (Lawrence et al., 2012); *Chlamydia* (Gondek et al., 2012) has been reported. These studies also indicated that $CD4^+$ T cells produced IFN-γ, a signature cytokine associated with protection against several intracellular pathogens. In response to the urgent need for an effective vaccine, in the current study, 27 *Ehrlichia ruminantium* recombinant proteins previously identified using needle infected animal PBMC (Pretorius et al., 2007; 2008; Sebatjane et al., 2010; Liebenberg et al., 2012) were assayed using tick infected, treated and challenged sheep as a source for immune PBMC. Ten proteins induced varied but significant production of IFN-γ and other cytokines in the PMBC tested. Interestingly, the use of PBMC derived from tick-infected animals resulted in significantly higher protein induced IFN-γ production than the results obtained previously when PBMC were obtained from needle challenged animals (Liebenberg et al., 2012).

In our study, IFN-γ responses were measured after 48 h incubation using an ELISPOT assay. It is well known that $CD4^+$ T cells and monocytes react faster than other cells, such as $CD8^+$ T cells, to produce IFN-γ responses and are the first line of defence against infection (reviewed by Whitmire, 2011; MacLeod et al., 2010). Other subtypes of T cells, which include γδ T cell subsets (Serre and Silva-Santos, 2013), may also produce this cytokine. To ascertain if $CD4^+$ T cells are the IFN-γ-producing lymphocytes that are required in the development of protective immunity against heartwater, an intracellular staining assay was performed and it was shown that $CD4^+$ Th1 T cells were producing IFN-γ when assayed with all the peptides that tested positive in IFN-γ ELISPOT assay. This could mean that these peptides contain immunodominant $CD4^+$ Th1 T cell epitopes within the selected antigens. It is important to note that the recombinant proteins induced higher IFN-γ production than the peptides. A possible reason could be that, the response to protein represents the sum of the responses to the individual peptides or this could indicate that peptide concentration requires further optimisation (Wherry et al., 2002).

A major challenge in heartwater is to understand the mechanisms that mediate protection in order to improve recombinant vaccine development. In the absence of an immunological correlate of protection, IFN-γ is often used as marker to identify promising vaccine candidates. It should be noted that IFN-γ expression alone is not always indicative of protection against *E. ruminantium* infection *in vivo* (Vachiéry et al., 2006). Thus, in addition to IFN-γ, other cytokine profiles induced by recombinant proteins and their peptides were determined and compared to those induced after stimulation with intact *E. ruminantium*. This was done in order to assist in designing a vaccine that mimics the immune response induced by the pathogen, which is characterised by production of IFN-γ, and other cytokines associated with adaptive immune responses and innate immunity (Liebenberg et al., 2012). Hence, it was investigated if the proteins as well as their peptides, that induced IFN-γ, were also capable of inducing other cytokines known to regulate inflammatory immune responses. The balance between pro- and anti-inflammatory networks is crucial to refine our knowledge and uses of the immune responses against *E. ruminantium*. Relative levels of cytokine mRNA expression in ovine PBMC stimulated by proteins and peptides were assessed using RT-qPCR assay. The cytokine profiles showed upregulation of pro-inflammatory cytokines, which include, IL-1α, IL-18, IL-12p40, TNF-α, iNOS, IFN-γ and GM-CSF but the cytokine mRNA levels varied between sheep. The levels of expression of a cytokine also varied between proteins and corresponding peptides and in general the proteins induced higher responses than their peptides. Of particular relevance is the fact that the proteins as well as their peptides selected in this study induced upregulation of Th1 cytokines. These play a role in the modulation of the immune responses, are growth factors for T lymphocytes and cytokines that activate neutrophils, macrophages, dendritic cells, recruit leukocytes to inflammatory sites and killing of pathogens. Therefore, addition of these antigens and peptides to a vaccine construct could contribute to the clearance of pathogens.

$CD4^+$ Th1 T cells play a central role in a functional adaptive immune response. They have been shown to be and validated as a major T cell subset that plays an important role in protection against *E. ruminantium*. The presence of $CD4^+$ T cells in β_2M-knockout mice (deficient for $CD8^+$ T cells), resulted in increased survival after an experimental *E. ruminantium* challenge (Byrom et al., 2000). Furthermore, it has been shown that PBMC from cattle rendered resistant to *E. ruminantium* challenge by vaccination with inactivated organisms contained *E. ruminantium*-specific, MHC class II-restricted, IFN-γ-producing, $CD4^+$ T lymphocytes (Totté et al., 1997). Moreover, Brown et al. (2002) has demonstrated an outer membrane antigen MSP2-specific $CD4^+$ T lymphocyte recognition of immunodominant epitopes of *Anaplasma marginale*. Moderzynski et al. (2016) also demonstrated that $CD4^+$ T

cells are as protective as $CD8^+$ T cells against *R*. *typhi*, if $CD4^+$ Th1 effector cells (Ec) are present in time to support bactericidal activity of phagocytes via the release of IFN-γ and other factors. It was also shown that $CD4^+$ helper T cells, but not cytotoxic T cells, orchestrate the rapid *E*. *chaffeensis* clearance in mice (Ganta et al., 2004). Previous *E. ruminantium* DNA vaccine formulations only focused on the activation of $CD8^+$ T cells due to the presence of an ubiquitin signal sequence. Nevertheless, given the fundamental role played by $CD4^+$ T cells in determining the functional status of both innate and adaptive immune responses, and the high number of specific $CD4^+$ T cell inducing peptides identified in this study, the inclusion of appropriate $CD4^+$ Th1 T cell epitopes may be essential for improvement of heartwater vaccine efficacy.

In addition, it has been generally accepted that CD45RO expression defines activated or memory T cells (Mahnke et al., 2013). In this study, $CD45RO^+$ was expressed on $CD4^+$ helper T cells when immune PBMC were stimulated with several peptides. Several tested peptides significantly induced $CD4^+CD45RO^+$ T cells from all sheep tested. It has been shown previously that $CD4^+$ T cells target P29 during *E. muris* infection and differentiated into IFN-γ producing Th1 effector/memory cells (Thirumalapura et al., 2013). The data obtained from this study has shown a remarkable increase of predominantly memory $CD4^+$ T cells when *E. ruminantium* peptides (p2540-6, p2540-16, p2540-19, p2540-20, p2540-21; p7140-6, p7140-7, p7140-12, p7140-13, p7140-20; p7320-8, p7320-9, p7320-21; p7350-9; p7620-2, p7620-12 and p8010-8) were used as stimulants.

The help provided by $CD4^+$ T cells is essential for the generation of a robust primary and memory $CD8^+$ T cell responses and protective immunity against various viral and bacterial infections (Hu et al., 2016). They promote the optimal expansion of cytotoxic $CD8^+$ T cells, maintain $CD8^+$ T cell memory, and communicate with innate immune cells (Phares et al., 2012; Green et al., 2013). Therefore, the combination of these epitopes with those that induce CD8 responses should be examined. Krawczyk et al. (2007) showed that memory $CD4^+$ T cells enhanced primary $CD8^+$ T cell responses.

The peptides reported in this study induced varied but significant activation of $CD4^+$ Th1 cells and production of cytokines in PBMC from most sheep tested. This variance can be expected because outbred animals were used for these assays and are probably a result of genetic differences (Babiuk et al., 2003; Lambkin et al., 2004; Kimman et al., 2007). It has been indicated previously that MHC class II molecules are highly polymorphic and different alleles vary in their peptide binding specificity (Groothuis et al., 2005; Sommer, 2006). As indicated previously, MHC typing done using ovine MHC II DRB1 highlighted the diversity between the

sheep used in this study (Thema et al., 2016). Each animal had one common allele (DRB1 *0201) with different combinations of the second allele and that could have contributed to the variability of immune responses among the individual sheep. This suggests that the peptides are most probably able to bind a number of different MHC class II alleles and that they are therefore ideal candidates for subunit vaccine design. This is very important as it could indicate that identification of MHC-allele specific T cell epitopes may not be enough for vaccine efficacy since epitopes selected must effectively cover the whole target population with its large diversity of alleles (Wilson et al., 2003).

Most importantly, several peptides that are reported in this study are conserved between 13 *E. ruminantium* strains. The selected peptides need to be conserved between heterologous strains or a mixture of peptides will be required for a vaccine to cover a variety of pathogen subtypes (reviewed by Skwarczynski and Toth, 2016). Genetic variation among *E. ruminantium* strains is one of the obstacles that hinders the progress in vaccine development (Raliniaina et al., 2010; Esemu et al., 2014). To address this obstacle, selection of epitopes that are most highly conserved among strains is critical. The logic behind this approach is that highly conserved epitopes have potential to induce more effective recall T cell responses and are able to protect against most strains. The results from this study are in agreement with other studies done where T cell epitopes of *M. tuberculosis* were highly conserved among different strains and were considered as promising candidates for vaccine design (Comas et al., 2010; Hui et al., 2015). Furthermore, conserved epitopes of influenza A virus were shown to induce protective immunity mediated by influenza specific B- and T cells (reviewed by Staneková and Varečková, 2010). In addition, vaccine-induced human $CD8^+$ T cells through conserved subdominant Pol epitopes were able to control HIV-1 replication *in vitro* (Ahmed et al., 2016). This knowledge complements our data that these epitopes may be useful in the design of a subunit vaccine.

This reductive selection method resulted in the identification of 23 Th1 immune response inducing peptides derived from ten proteins that were predicted to be membrane or surface associated, exported or proteins with unknown function. Membrane or exported proteins are regarded as good vaccine candidates as they contain within their sequence an export signal. In the case of intracellular bacteria, export signals allow the bacterial proteins to be secreted into the host cell cytoplasm environment. Additionally they may become associated with the host cell cytoplasmic membrane/cell wall (reviewed by Sánchez et al., 2008). From here, they are well positioned to modify host responses. On the other hand, this association with the cytoplasm and membranes may expose the intracellular infection to the host making them ideal vaccine candidates. In this study, $CD4^+$ Th1 T cell epitopes derived from these

membrane or exported proteins protein were identified. Most importantly, the peptides gave positive results in IFN-γ ELISPOT, $CD4^+CD45RO^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ T cells assays. In addition, different peptides induced different cytokines as shown by the upregulation of pro-inflammatory cytokines, which include, IL-1α, IL-2, IL-12p40, TNF-α, iNOS, IFN-γ and GM-CSF.

In conclusion, 23 epitopes from ten proteins were identified by recall immune responses and characterised by activation of memory $CD4^+$ Th1 T cells and different Th1 cytokine production profiles. It is therefore possible that the inclusion of these epitopes to a vaccine formulation will improve protection obtained from the vaccine.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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