Ehrlichia ruminantium antigens and peptides induce cytotoxic T cell responses *in vitro*

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Abstract

Since $CD8^+$ T cells play an important role in resistance to infection with heartwater, effective vaccines against this disease will likely require identification of antigens that contain $CD8^+$ T cell epitopes responsible for cytotoxic T lymphocyte (CTL) responses. With the use of the fluorescent antigen-transfected target cell (FATT)-CTL assay, IFN-γ ELISPOT and flow cytometry, peptides that induce CTL, proliferation of CD8+T cells and IFN-γ production were identified as possible target antigens for vaccine development. Of particular relevance was the finding that different peptides from different antigens were able to elicit varied cytotoxic activities by immune peripheral blood mononuclear cells (PBMC) from heartwater immune tick-infected sheep. Several peptides derived from Erum0660, Erum2330, Erum2540, Erum2580 and Erum5000 induced CTL in immune sheep PBMC. Peptide Erum2540-6 was the only peptide that induced significant CTL, $CD8^+CD45RO^+$ and $CD8^+IFN\text{-}\gamma^+$ by PBMC from all three sheep, and Erum2540 and p2540-20 induced the highest % CTL response in all three outbred sheep. These results suggest that these epitopes may be of major importance in heartwater recombinant vaccine development.

Keywords: *E. ruminantium*, antigens, $CD8^+$ T cells, CTL epitopes, FATT-CTL

Highlights:

- Optimised FATT-CTL assay for the first time for sheep PBMC
- FATT-CTL assay was used to identify peptides that induced CTL
- Peptides from different antigens induced varied CTL responses in immune PBMC
- Peptides may be included in heartwater recombinant vaccine development

Abbreviations: effector cells (Ec), fluorescent antigen-transfected target cell (FATT)-CTL assay, cytotoxic T lymphocyte (CTL)

1. INTRODUCTION

Despite several attempts to develop improved vaccines for the control of heartwater, the blood vaccine is still in use, emphasising the urgent need for an improved, safer and effective vaccine. Effective vaccine strategies against *E. ruminantium* infection depend, in part, on understanding the host protective immune responses. Since *E. ruminantium* is an obligate intracellular rickettsial agent, cell-mediated immune (CMI) responses are expected to play an important role in protection [1]. Protective immunity against *E. ruminantium* is characterised by the proliferation of $CD4^+$, $CD8^+$, and γδ T cells as well as expression of Th 1 cytokines including IFN-γ, TNF-α, TNF-β and IL-2 [2]. Of particular relevance to this present study is that IFN-γ has been shown to inhibit *E. ruminantium* growth in endothelial cells *in vitro* [1, 3]. Both $CD4^+$ and $CD8^+$ T cells are IFN-γ-producing T lymphocytes that are required in the development of protective immunity against heartwater. Of the two major T cell subsets, the $CD8^+$ T cells play a critical role in intracellular bacterial clearance. For example, during tuberculosis and parasitic protozoa infections, genetically altered mice lacking $CD8^+$ T cells are more susceptible to infection than are immunocompetent wild type mice or mice lacking $CD4^+$ T cells [4, 5]. In addition, Byrom et al. [6] demonstrated that $CD4^+$ T cell KO mice were able to resist *E. ruminantium* challenge following infection and treatment suggesting an important role for $CD8^+$ T cells in protection. Although $CD8^+$ T cells seem to be the main effector cells involved in protection against several intracellular pathogens, the mechanisms by which $CD8^+$ T cells participate in the control of heartwater infection are still not completely understood.

Much effort has been spent on the identification of proteins that induce strong cell-mediated immune responses characterised by $CD4^+$ or $CD8^+$ T cell responses. To date in our laboratory several *E. ruminantium* proteins have been characterised [7, 8, 9, 10, 11]. These studies have shown that T cells induce IFN-γ production in immune sheep or bovine peripheral blood mononuclear cells (PBMC) but assays done have not included the cytotoxic T cell assay to show that $CD8^+$ T cells induce lysis of target cells. The identification of proteins and the corresponding peptides that induces cytotoxic T lymphocyte (CTL) would be of great benefit for the design and development of a multi-epitope DNA vaccine. In previous studies on *Mycobacterium tuberculosis* (Mtb), a lot of work has been done to identify CTL epitopes of Mtb antigens. The main focus was based on secreted proteins such as the early secreted antigen target 6 kDa (ESAT-6) [12] and antigen 85B (Ag85B) [13]. Furthermore, CTL epitopes derived from culture filtrate protein 21 kDa (CFP21) and region of differentiation (RD) antigens [14, 15, 16] were identified.

The radioactive chromium (^{51}Cr)-release assay is the most popular assay for evaluating cell-mediated cytotoxicity. However, it has several drawbacks, which include biohazard and disposal problems associated with radioisotope usage [17]. To determine the cytotoxicity of $CD8^+$ T cells the human fluorescent antigen-transfected target cell (FATT)-CTL assay was adapted for use in sheep to identify the epitopes that induce CTL for inclusion in a multi-epitope vaccine. The FATT-CTL assay combines non-radioactive quantification of target cell killing with an efficient plasmid transfection technology to achieve antigen expression in target cells [18]. It measures antigen-specific cell mediated cytotoxicity and is a convenient alternative to the classic radioactive Cr^{51}-release assay. It is also preferred in our study, since the assay indirectly tests if protein is expressed, cleaved by the proteasome and presented on MHC I following transfection with the pCMViUBs mammalian expression vector.

In previous *E. ruminantium* vaccine development studies in our laboratory, 14 genes were selected as vaccine candidates based mainly on their ability to induce IFN-γ [8, 9, 10, 11]. They were cloned in the pCMViUBs mammalian expression vector, that targets activation of cytotoxic T cell responses [7], and tested in sheep. However, only a cocktail of four genes (Erum2540, 2550, 2580, 2590) induced 100% protection against needle challenge. The aim of this study was to identify which of these 14 gene products and epitopes induced upregulated IFN-γ producing $CD8^+$ T cells and CTL responses in PBMC from tick challenged immune sheep. The FATT-CTL assay was therefore applied to detect antigen-specific cell-mediated cytotoxicity in sheep.

2. MATERIALS AND METHODS

2.1 Animals and Ethical statement

Merino sheep (s6819, s6821, s6822 and s6823) aged between 8 and 12 months were obtained from a heartwater free region (Warden, Free State Province) in South Africa and confirmed negative for *E. ruminantium* using the pCS20 qPCR [19]. 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).

2.2 Immunological assays

2.2.1 Inoculation of animals as source of immune mononuclear cells

To mimic natural infection, ticks experimentally infected with the Welgevonden strain [11] were fed onto three sheep (s6821, s6822, s6823). Briefly, uninfected *Amblyomma hebraeum*

nymph ticks were infected by feeding on a sheep (s6819) that had been infected intravenously with *E. ruminantium* Welgevonden blood stabilate. 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 [19].

2.2.2 Purification of PBMC

PBMC were purified from sheep blood under sterile conditions as described previously [10]. 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.

2.2.3 Cytotoxicity analysis

2.2.3.1 Cloning of E. ruminantium genes in a GFP containing mammalian expression vector

Overlap extension polymerase chain reaction (OE-PCR) was used to clone a *gfp* gene originating from Vitality phrGFP into the into pTandem plasmid (Novagen®), which contains an internal ribosomal entry site (IRES) sequence (SFigure 1, Supplementary data). Sequencing confirmed that the sequence was correct. The IRES_GFP fragment was then amplified from this plasmid with with primers: pIRES-GFP II F (5'-ATGGGCGGTAGGCGTGTA-3') and pIRES-GFP II R (5'-ATGCAGTCGTCGAGGAATTG-3'). The IRES_GFP fragment was then cloned in each of the 14 pCMViUBs vectors containing an *E. ruminantium* gene [7] using an OE-PCR cloning method [20]. Briefly, inserts were amplified with Mega primers, using 2 x master mix of Q5 high fidelity polymerase (NEB), 500 nM of each primer and 50 ng template DNA. The primers were UB/Tandem F (5'-CCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCCACTAAGTGAT TAACCTCAGGACTAG-3') and UB/Tandem R (5'-CACCCCCCTCCACCCCATAATATTATA GAAGGACGGGGTTATGCTAGTTACATATGG-3'). The OE-PCR amplification was done by mixing the insert and vector at a 50:1 insert: vector ratio, using 2.5 units (U) *TaKaRa LA Taq polymerase* (TAKARA) and 1 U Phusion taq (NEB), 1 x *TaKaRa LA Taq polymerase* buffer, 0.4 mM dNTPs and 3 mM $MgCl_2$. The PCR conditions were: 98°C (denature), 1 min followed by 20 cycles of 98°C, 10 s; 72°C 5 min followed by a final extension step of 72°C for 10 min.

After this PCR was completed, 10 U *DpnI* enzyme (Thermo Scientific™) was added to the reaction and incubated for an additional 120 min at 37°C; followed by inactivation at 85°C for 10 min to digest parental plasmid. The amplified plasmid was transformed into *E. coli* TOP10 cells (Invitrogen) and the resulting putative recombinant clones with the correct insert size were selected and sequenced. Endotoxin free plasmid DNA (pDNA), required for the CTL assay, was prepared using the Endofree Plasmid Maxi kit (Qiagen) according to the manufacturers recommendations.

2.2.3.2 FATT-CTL assay

The FATT-CTL assay was carried out as described previously, with minor modifications [21, 22]. Firstly, the method was optimised for use with sheep PBMC. Briefly, target cells (PBMC) were electroporated with either empty pIRES- hrGFP II vector (negative control) or with 14 different pCMViUBs_gene_IRES-hrGFP vectors. To optimise the assay, PBMC (1×10^7 cells/ml) were washed with PBS and resuspended in 1000 µl electroporation buffer (Bio-Rad). Cells were divided into aliquots and each aliquot of cells was electroporated with 4 µg of the empty pIRES- hrGFP II vector at different voltage settings (150 V, 200 V, 250 V and 300 V) with constant capacitance at 1000 µF and ∞Ω resistance. Using pCMViUBs_Erum2540_IRES-hrGFP, the incubation time and effector cell: target cell ratio were also optimised for the assay. The optimised FATT-CTL assay was done as follows: Triplicate wells of effector cells at 5-50 x 10^6 cells/ml were stimulated with 14 *E. ruminantium* recombinant proteins and several overlapping synthesised 16 mer peptides at a final concentration of 10 µg/ml. These genes were cloned in the pET102 TOPO vector as described previously [10]. For the present study, the proteins were freshly expressed in *E.coli,* precipitated using acetone and checked on SDS-PAGE and Western blot, as described previously [9]. Target cells were seeded in triplicate in a 48 well plate at 5 x 10^5 cells per well. These target and effector cells were incubated for 24-96 h at 37°C in a humidified 5% CO_2 incubator before the effector cells were loosened and added to the target cells at effector cell: target cell ratios of 1:1. Target and effector cells were included individually as controls for monitoring GFP expression. Empty vector was included as a control to determine if lysis was induced by the plasmid itself (transcription control) or if the recombinant protein induced lysis. The percentage (%) lysis was determined by acquiring the number of GFP positive cells using a cytomics FC 500 flow cytometer® with Kaluza 1.2 software (Beckman Coulter). To reduce the number of calculations, the number of GFP positive cells were measured and converted to events/second, since concentration (events/µl) cannot be measured on the FC500 flow cytometer. Proteins and peptides were tested at more than one time point. For example, peptides for Erum2540 were used for optimising the assay and it was done at three time points.

Representative FATTC-CTL assay histograms for Erum2540 peptide 8 showing the total number of GFP positive events for all tests and controls used are shown in SFigure 2. The % lysis was calculated as shown below in Table 1. The values above 6% were considered positive.

Table 1. Example of calculations used to determine the % lysis, done similarly to Faber et al. (2016) with some modifications. To reduce the number of calculations, the number of GFP positive cells were measured and converted to events/second, since concentration cannot be measured on the FC500 flow cytometer. As an example of the calculations done for one sample, the % lysis induced by peptide p2540-6 (P6) in PBMC from s6821 are shown. The average % lysis induced by PBMC from three sheep were calculated for each peptide tested and listed in Table 2.

	# Ave GFP positive cells	Ave Time (s)	Ave cells/s	% Lysis
pGFP_empty Target only	4446±698	212±3	21	
pGFP_empty + Effector P6[a]	878±136	47±2	19	10
pUB2540 Target only	8053±0	156±11	31	
pUB2540 + Effector MED[b]	6792±0	152±3	45	0[e]
pUB2540 + Effector P6[c]	676±50	48±2	14	55

[a]rProtein control % Lysis =100* [(Target only pGFP_empty (events/s)) - (pGFP_empty + E P6 (events/s))]/(Target only pGFP_empty (events/s)).
[b]Expression control % Lysis =100* [(pUB2540 Target only (events/s)) - (pUB2540 + Effector MED (events/s))]/(pUB254 Target only (events/s)).
[c]Test sample % Lysis = 100* [(pUB2540 Target only (events/s)) - (pUB2540 + Effector P6 (events/s))]/(pUB2540 Target only (events/s)).
[e]Negative values are indicated as 0 % Lysis.

2.2.4 Cell surface staining of the effector cells

Effector cells stimulated with antigens for 72 h at 37°C were used for analysis and were seeded in triplicate. 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: 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 PN buffer (1 x PBS containing 0.5% FBS and 0.2% sodium azide). Following washing, the corresponding secondary antibodies: goat anti-mouse IgG1-PE and goat anti-mouse IgG3-FITC (Serotec) were added at a dilution of 1:30 and 1:10 respectively. Immunoglobulin isotype controls (MCA928, IgG1, MCA2063, IgG3 (AbD, Serotec, Biorad)) were included in order to test nonspecific binding of the secondary antibodies. All incubations were carried out 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.

2.2.5. Intracellular IFN-γ staining

Immune PBMC purified from three sheep (s6821 s6822 and s6823) were used for intracellular IFN-γ staining using the BD Cytofix/Cytoperm™ Kit (BD Biosciences) according to the manufacturer's instructions. 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) 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.

2.3 Selection and synthesis of *Ehrlichia ruminantium* vaccine candidate epitopes

Proteins with positive IFN-γ were selected for T cell epitope mapping. Peptides of 16-mer and overlapping by 8 amino acids, spanning the whole protein, were synthesized by Genscript and the purity of the peptides was >98% as analysed by high-performance liquid chromatography. However, a number of peptides could not be synthesised due to high hydrophobicity. The peptides were either dissolved in water or 100% dimethyl sulfoxide to 1 mg/ml and stored at - 70°C. Peptides were further diluted to 100 µg/ml in complete medium prior to use in the same immune assays as listed above. Epitope prediction was done for Erum2540, 2580, 2330, 5000 and 0660, using the Bovine MHC class I alleles from the IEDB Analysis Recourse database (http://tools.immuneepitope.org/mhci). Only IEDB recommended low percentile rankings below 2.19 were considered as good binders and were included in the analysis. Sequence alignments were done in CLC Genomics Workbench 7.5 from Qiagen (http://www.clcbio.com/products/clc-genomics-workbench/).

2.4 Statistical analysis

The Student's t-test was used to determine statistical differences observed between a stimulated positive response compared to the negative unstimulated control of each experimental repeats and three biological repeats. Differences with p values ≤ 0.05 were considered significant.

3. RESULTS

3.1. Antigen, vector preparation and optimisation of the CTL assay

3.1.1 Cloning of E. ruminantium genes in mammalian expression vector

E. ruminantium genes were successfully cloned in a mammalian expression vector and transfect into target cells. A total of 14 recombinant pCMViUBs_Erum-gene plasmids (Erum0660, Erum1050, Erum1150, Erum2300, Erum2330, Erum2540, Erum2550, Erum2580, Erum2590, Erum3750, Erum5000, Erum5400, Erum5420 and Erum7360) previously used in animal trials were selected and the IRES_hrGFP fragment was successfully cloned into them. Sequencing confirmed that the genes were in frame with ubiquitin (UB) and that the IRES and GFP genes had the correct sequence. All the proteins described were expressed previously [10].

3.1.2 Optimisation of transfection conditions

Optimisation of transfection conditions for sheep PBMC was done using an empty pIRES-hrGFP II plasmid. DNA was successfully introduced into PBMC using the Bio-Rad gene pulser transfection apparatus. Expression of GFP could be detected at both 24 h and 48 h and showed that ~45% of the cells were GFP positive (Figure 1). Thus, optimum conditions used in all subsequent FATT-CTL assays were 4 µg pDNA electroporated at 300V, 1000µF and ∞Ω for 48 h.

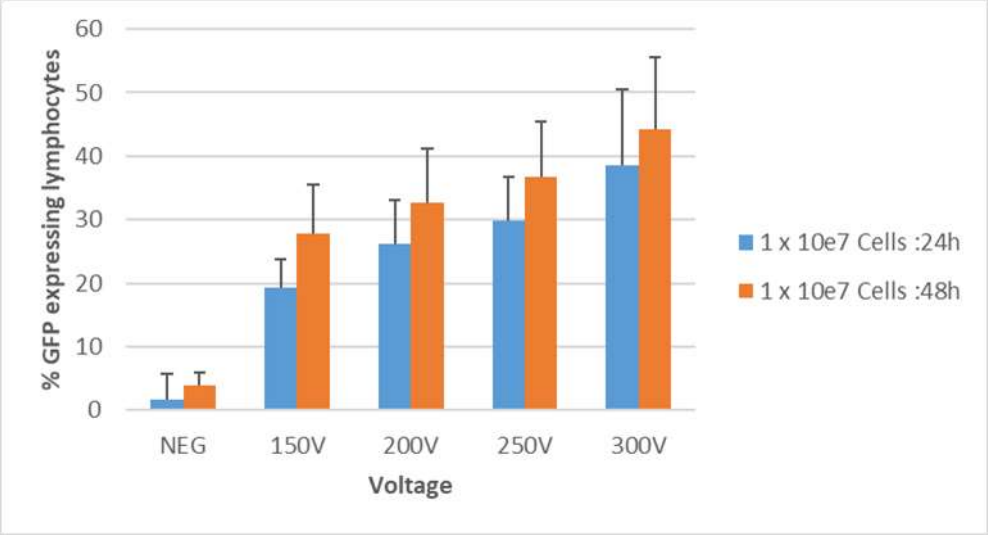

Figure 1. The % GFP positive cells after electroporation with 4 µg pGFP at different voltage settings with constant 1000 µF and ∞Ω. The optimum conditions for detecting expression of GFP is at 48h with DNA electroporated at 300V.

3.1.3. Optimisation of the FATT-CTL assay for sheep PBMC

The FATT-CTL assay was first optimised using the Erum2540 gene. An optimised non-radioactive CTL assay was tested using immune sheep PBMC to determine if Erum2540 induces cytotoxicity. The effector cells were stimulated with intact crude *E. ruminantium* antigen for 24, 48, 72 and 96 h. Target cells were electroporated with 4 µg of either the empty pIRES-hrGFP II plasmid as negative control, or the pCMViUBs_Erum2540_IRES_hrGFP using the optimum electroporation conditions. After 24, 48, 72 and 96 h incubation the effector and target cells were mixed at 1:1, 1:2, 1:3 ratios and, after an overnight incubation, the % lysis over time was determined. Optimum lysis was detected after 72 h incubation (Figure 2), protein concentrations between 1 and 10 µg/ml and at an effector cells: target cells ratio of 1:1. Testing % lysis at different ratios and 72h incubation confirmed that a ratio of 1:1 and a protein concentration of 1 µg/ml gave the highest % lysis (Figure 3). Since high throughput analysis was done, and considering the limited amount of PBMC collected at a time point from one sheep, a ratio of 1:1 was selected for all subsequent analysis. Optimum conditions that were used in all subsequent assays were a 1:1 effector cells: target cells ratio, 1-10 µg/ml protein or peptide and 72 h incubation.

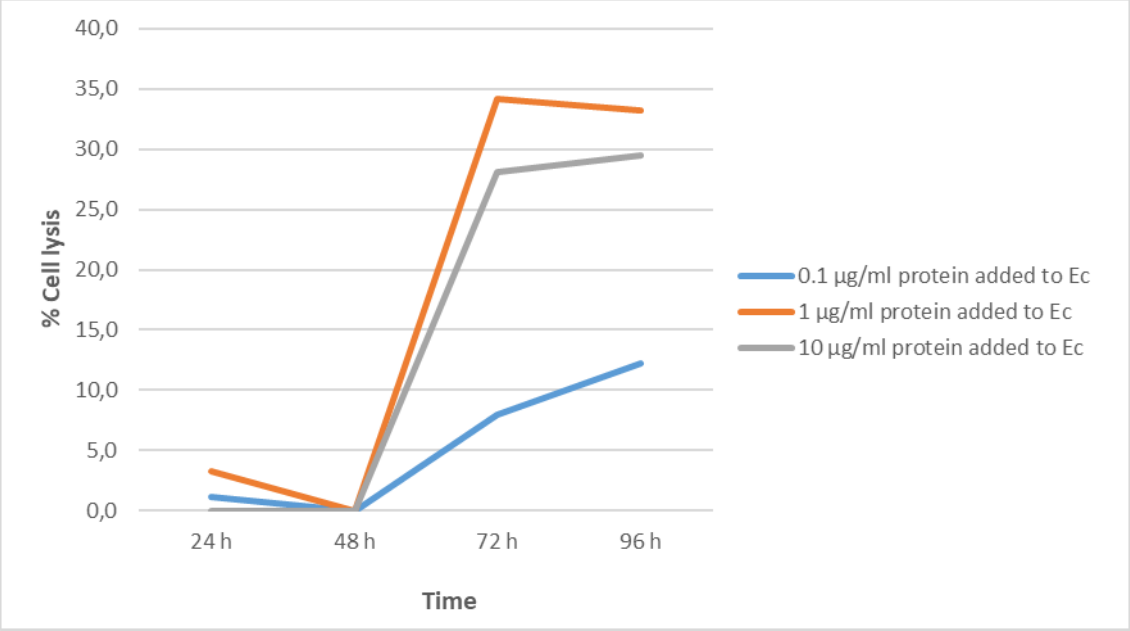

Figure 2. Optimisation of the incubation time for FATT-CTL assay using different antigen concentrations, 0.1 µg/ml (blue line), 1 µg/ml (red line) and 10 µg/ml (grey line). effector cells (Ec) and target cells were incubated at a ratio of 1:1 for 24, 48, 72 and 96 h incubation time and then harvested and the % lysis calculated determined over time. Both the rProtein control and expression control were negative at all times tested.

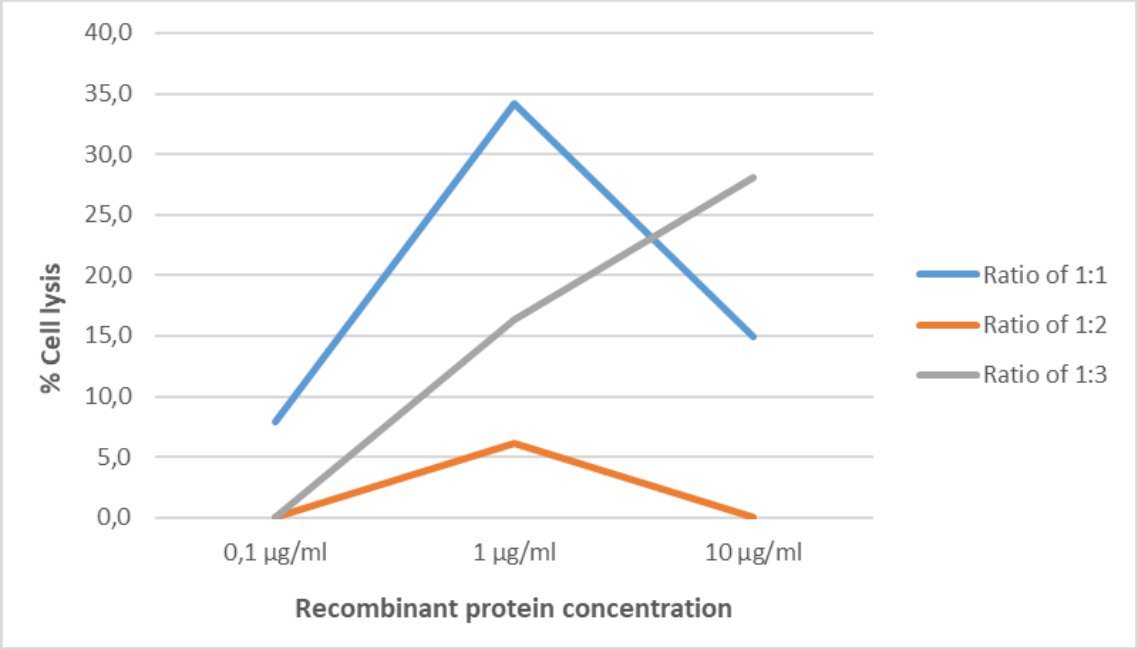

Figure 3. Optimisation of the target to effector cell ratio at 72 h incubation time for FATT-CTL assay. Effector and target cells were incubated at a ratio of 1:1 (blue line), 1:2 (red line) and 1:3 (grey line) at three different protein concentrations, 0.1, 1 and 10 µg/ml. Cells were harvested and the % lysis calculated. Both the rProtein negative control and expression control were negative at all times tested.

3.1.4. *E. ruminantium* proteins that induce CTL, memory CD8 and $CD8^{+}IFN\text{-}\gamma^{+}$ responses

The optimised FATT-CTL assay was used to detect *E. ruminantium* antigen-specific cell-mediated cytotoxicity in immune sheep PBMC. PBMCs were obtained from three immune sheep s6821, s6822 and s6823. Effector cells were stimulated with 14 recombinant proteins, previously characterized (Manuscript in preparation) and Erum5400 (negative protein control) Target cells were electroporated with different plasmids containing the *E. ruminantium* genes that corresponds to each protein and an empty vector control. The assay was done in triplicate for each antigen/peptide for each sheep (experimental repeats). Average results are shown and compared between all three sheep (biological repeats) as shown in Table 2. Following the CTL assay, using the optimised parameters stated above, only four recombinant proteins induced CTL. CTL responses specific to Erum2540 was obtained for all sheep tested and was statistically significant between the biological repeats (between sheep), while proteins Erum5000 induced toxicity in PBMC from two sheep (s6821 and s6822) and Erum0660,Erum2330 and Erum2580 induced CTL responses only in one sheep (Table 2). Moreover, the proteins, Erum1050, Erum1150, Erum2300, Erum2550, Erm2590, Erum3750, Erum5400, Erum5420 and Erum7360 did not induce noticeable CTL responses.

Table 2. Summary of the $CD8^+$ T cell responses induced by *E. ruminantium* recombinant proteins *in vitro* in PBMC from infected tick challenged sheep. These responses include induction of cytotoxicity, memory CD8 and $CD8^+IFN\text{-}\gamma^+$. Values in bold indicate significance between experimental and biological repeats (between sheep) and proteins in bold were selected for further epitope analysis.

Recombinant proteins	**Function**	**FATT-CTL assay (Ave % Lysis induced)**				**$CD8^+CD45RO^+$ T cells (Ave % increase above medium background)**				**$CD8^+IFN\text{-}\gamma^+$ T cells (Ave % increase above medium background)**			
		s6821	**s6822**	**s6823**	**Ave ± Stdev**	**s6821**	**s6822**	**s6823**	**Ave ± Stdev**	**s6821**	**s6822**	**s6823**	**Ave ± Stdev**
Erum2540	Exported	**54**	**21**	**20**	**32±19.4**	**4**	**5**	**8**	**6±1.7**	**6**	**6**	**4**	**5±1.2**
Erum2550	ATP-binding protein part of ABC transporters	0	0	0	0	0	**6**	0	2±3.5	0	**2**	**6**	3±3.1
Erum2580	Periplasmic solute binding protein (SBP)	0	4	**31**	12±14.0	0	**6**	**2**	3±3.3	0	**2**	**1**	1±1.0
Erum2590	ATP-binding protein part of ABC transporters	0	0	0	0	0	0	**4**0	1±2.3	0	0	**4**	1±2.3
Erum7360	Membrane LMW	0	0	0	0	**2**	**2**	**3**	**2±0.5**	**4**	**5**	**4**	**4±0.6**
Erum5420	GTP-binding ERA	1	1	1	1±0	**6**	**4**	**3**	**4±0.8**	**6**	**7**	**7**	**7±0.6**
Erum3750	Unknown	1	1	1	1±0	**3**	**10**	**10**	**8±3.3**	**1**	**3**	**3**	**2±1.2**
Erum2330	Exported	**50**	0	4	18±27.8	**3**	**33**	**3**	13±14.1	**2**	**5**	**3**	**3±1.2**
Erum2300	Membrane	1	0	1	1±0.6	**1**	**8**	**3**	4±2.9	**2**	**2**	**2**	**2±0**
Erum1150	Unknown	1	0	1	1±0.6	**15**	**4**	**3**	7±5.4	**1**	**3**	**3**	**2±1.2**
Erum1050	Integral membrane	1	0	1	1±0.6	**8**	**4**	**4**	**5±2**	**2**	**2**	**1**	**2±0.6**
Erum0660	Unknown	**40**	0	1	14±22.8	**2**	**4**	**2**	**3±0.9**	**2**	**3**	**1**	**2±1.0**
Erum5000	Exported	**9**	**12**	0	7±6.2	**14**	**223**	0	79±125	**4**	1	**1**	2±1.7
Erum5400 (Neg. protein)	Unknown	0	0	0	0	0	0	0	0	0	1	0	0.2±0.6
Ag+	Crude *E. ruminantium* antigen	-	-	-	-	**6**	**88**	**3**	32±48.2	**2**	**2**	**3**	**2±0.6**
ConA	Positive control	-	-	-	-	**32**	**53**	**30**	**38±12.7**	**10**	**5**	**4**	**6±3.2**

Lysis, significant % increase of memory $CD8^+$ T cells and IFN-γ producing 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.
(-) not determined.

In order to determine whether the proteins that induced lysis also induced the activation of memory $CD8^+$ T cells, the effector cells were double stained for expression of CD45RO by $CD8^+$ T cells. The results showed that significant memory $CD8^+$ T cells were induced by six of the recombinant proteins tested using PBMC from three immune sheep (Table 2). Similarly, an intracellular staining assay was done to determine whether the induced $CD8^+$ T cells were also IFN-γ producing cells. All proteins that induced memory $CD8^+$ T cells were also IFN-γ producing $CD8^+$ T cells (Table 2). The immune responses obtained were significantly higher than the negative protein

3.1.5. *E. ruminantium* peptides that induce a CTL response

Since Erum2540, Erum2580, Erum2330, Erum5000 and Erum0660 induced CTL responses, memory $CD8^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells, individual 16 mer peptides (overlapping by 8 amino acids) derived from these proteins were tested for their ability to induce cytotoxicity. Peptides from Erum2550 and Erum2590 were also included in the assays because these proteins are part of the 1H12 vaccine that induced protective immunity in the past. Peptide sequences and the *in silico* predicted epitopes using bovine MHC I alleles are listed in STable 1. Prediction results showed that there were epitopes predicted at most amino acids in each protein (STable 1 and SFigure 4) and that more than one MHC I allele can bind the same epitope (SFigure 5). The C terminus peptides of Erum2540 (P15-21) each contained more than one predicted epitope (STable 1 and SFigure 5). PBMC obtained from *E. ruminantium*-tick infected sheep s6821, s6822 and s6823 were used. Effector cells were stimulated with individual peptides and target cells were electroporated with plasmids containing the different corresponding *E. ruminantium* genes using the optimum conditions described above. The average lysis obtained from a target to effector cell ratio of 1:1 is shown (Table 3). Only peptides that are conserved between 13 *E. ruminantium* strains were tested to ensure that they are able to protect against most strains. Only three peptides from Erum0660 (p0660-12, p0660-25/26 and p0660-42) induced positive cell lysis which ranged between 14-25% in one sheep (s6822). Regarding, Erum5000, peptides p5000-6/7 and p5000-10 induced positive cell lysis in all three sheep tested. Interestingly, 10 peptides derived from Erum2330 induced CTL responses in all three sheep tested, while seven peptides induced varied cell lysis in two sheep. Of these, significant results were obtained for p2330-4, p2330-7 and p2330-23/24. Individual peptides (p2540-6, p2540-8, p2540-12, p2540-18, p2540-20 and p2540-21) derived from Erum2540 induced the highest CTL responses in all three immune sheep PBMC tested (Table 3). Although other Erum2540 peptides induced cytotoxic activities, the responses were only positive in two sheep tested (s6821 and s6823). Peptide p2540-20 induced the highest average lysis of all the peptides tested. Moreover, peptides derived from Erum2550 (p2550-

Table 3. FATT-CTL assay of PBMC from three sheep stimulated with peptides of Erum0660, Erum2330, Erum2540, Erum2550, Erum2580, Erum2590 and Erum5000 at a concentration of 10 µg/ml. Effector cells and target cells were mixed at a ratio of 1:1. The average % lysis of each individual sheep, average % increase of $CD8^+$ T cells for each individual sheep as well as the average for the group are indicated. Only positive results are shown.

Peptides	FATT-CTL assay: Ave % Lysis induced				$CD8^+$ T cells Ave % increase above medium background			
	s6821	s6822	s6823	Ave ± Stdev	s6821	s6822	s6823	Ave ± Stdev
p0660-12	0	14	0	5±8.1	1	0	1	1±0.6
p0660-25/26	0	18	0	6±10.4	2	0	1	1±1
p0660-29/30/31	0	5	0	2±2.89	2	1	1	**1±0.6**
p0660-42	0	25	0	8±14.4	3	1	1	**2±1.2**
p2330-1	33	7	11	**17±14**	11	79	7	32±40.5
p2330-2	21	13	0	11±10.6	14	59	8	27±27.9
p2330-3	10	1	22	11±10.5	0	59	3	21±33.2
p2330-4	10	14	2	**9±6.1**	15	44	6	22±19.9
p2330-5	12	58	6	25±28.5	2	40	9	17±20.2
p2330-6	20	46	10	**25±18.6**	3	46	1	17±25.4
p2330-7	21	3	13	**12±9.0**	0	81	4	28±45.7
p2330-8	26	14	0	13±13.0	13	53	2	23±26.8
p2330-9	21	23	15	**20±4.2**	6	84	0	30±46.9
p2330-10	18	22	12	**17±5.0**	4	46	2	17±24.8
p2330-11	30	19	20	**23±6.1**	0	63	6	23±34.8
p2330-12	30	20	26	**25±5.0**	0	60	1	20±34.8
p2330-19	28	15	8	**17±10.2**	12	53	0	22±27.8
p2330-20	24	13	20	**19±5.6**	2	50	0	17±28.3
p2330-21	31	18	10	**20±10.6**	7	48	0	18±25.9
p2330-22	33	19	20	**24±7.8**	4	86	6	32±46.8
p2330-23/24	0	32	14	15±16.0	3	71	2	25±39.6
p2540-6	55	25	35	**38±15.3**	0	10	5	5±5.0
p2540-8	44	39	17	**33±14.5**	11	2	6	**6±4.5**
p2540-12	51	40	24	**38±13.5**	11	7	9	**9±2.0**
p2540-15	53	0	25	26±26.7	10	6	8	**8±2.0**
p2540-16	34	1	33	**23±19.1**	11	16	13	**13±2.5**
p2540-18	53	38	18	**36±17.8**	14	16	15	**15±1.0**
p2540-19	57	0	1	19±32.6	10	14	11	**12±2.1**
p2540-20	72	38	9	**40±31.3**	12	6	9	**9±3.0**
p2540-21	58	19	9	29±25.7	14	9	12	**12±2.5**
p2550-19	7	0	0	2±4,0	0	57	6	21±31.3
p2580-8	0	0	4	1±2,3	15	34	22	**24±9,6**
p2580-10	0	0	32	11±18,5	4	60	13	26±30.1
p2580-12	29	25	17	**24±6,1**	7	81	11	33±41,6
p2580-33	25	0	0	8±14,4	0	36	18	18±18
p2580-34	26	0	0	9±15,0	14	44	5	21±20,4
p2580-35	40	4	0	15±22,0	18	55	18	**30±21,4**
p2590-23	8	0	0	3±4,6	2	55	18	25±27,2
p5000-6/7	39	37	22	**33±9.3**	0	65	0	22±37.5
p5000-8/9	2	44	5	17±23.4	5	65	0	23±36.2
p5000-10	19	47	11	**26±18.9**	8	61	0	23±33.2
Neg.peptide	0	0	0	0	0	0	1	0±0.6
Empty vector control	0	0	0	0	-	-	-	-
Expression control	0	0	0	0	-	-	-	-

Positive lysis above 6 percent is indicated in red, a positive % increase of $CD8^+$ T cells that is two times higher than the negative peptide is indicated in blue. Samples with $p \leq 0.05$ values (Student's t-test) are considered significant and shown in bold. The average values are expressed as means ± SD. Empty vector control and Expression control were also added as negative controls for the FATT-CTL assay.

19), Erum2580 (p2580-10, p2580-33, p2580-34, p2580-35) and Erum2590 (p2590-23) induced CTL responses in one sheep with the exception of Erum2580 (while p2580-12), which induced positive and significant CTL responses in all three sheep. Many of the predicted epitopes tested negative in the immune assays while some peptides that tested positive did not contain a predicted epitope (STable1).

Furthermore, in order to determine the phenotype of the responding effector cells responsible for peptide-specific cytotoxicity, PBMC from three sheep (s6821, s6822 and s6823) were used. A sample of the effector cells was stained with fluorochrome-labeled anti-CD8 antibodies and flow cytometric analysis done. All peptides induced an increase of $CD8^+$ T cells between one to three of the sheep (Table 3). Peptides p2540-8, p2540-12, p2540-15, p2540-16, p2540-18, p2540-19, p2540-20, p2540-21, p2580-8 and p2580-35 induced significant $CD8^+$ responses (p values ≤ 0.05). Most importantly, all assays were repeated at more than one time point to show the reproducibility of results.

3.2. Peptides that stimulate $CD8^+CD45RO^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells

After stimulation of PBMC with peptides that induced cytotoxicity, the cells were stained for expression of memory $CD8^+$ T cells and for intracellular staining of IFN-γ (See SFigure 3 for representative histograms). Isotype controls were included in the assays as negative controls to distinguish the non-specific background signal from specific antibody signal (See SFigure 3 for representative histograms). The top five inducers are shown in Table 4. Only p2540-6 showed an increase of $CD8^+CD45RO^+$ T cells in all three sheep tested. In addition, an increase in memory $CD8^+$ T cells in at least two sheep (s6822 and s6823) was observed for peptides p2540-16, p2540-19, p2540-20, and p2540-21. The data varied between animals.

As shown in Table 4, peptides p2540-6, p2540-16, and p2540-19, induced % increase of $CD8^+IFN\text{-}\gamma^+$ T cells in three sheep, which was significantly different ($p \leq 0.05$) compared to the negative control. Overall, these results demonstrated that these peptides induced $CD8^+$ T cells to produce IFN-γ and CTL responses were activated.

Table 4. The five peptides that induced $CD8^+CD45RO^+$ and $CD8^+IFN\text{-}\gamma^+$ T cell as indicated by phenotypic analysis of PBMC from immune sheep (s6821, s6822, and s6823).

Peptides	$CD8^+CD45RO^+$ T cells (Ave % increase)			$CD8^+IFN\text{-}\gamma^+$ T cells (Ave % increase)		
	s6821	**s6822**	**s6823**	**s6821**	**s6822**	**s6823**
p2540-6	**44±1.1**	**96±0.3**	**52±0.5**	**5±0**	**3±0.2**	**3±0.1**
p2540-16	0	**87±0.5**	**20±0**	**3±0.4**	**2±0.3**	**3±0.5**
p2540-19	0	**62±0.5**	**30±0.2**	**2±0.1**	**2±0.5**	**3±0.5**
p2540-20	0	**73±0.1**	**34±0.4**	**3±0.3**	2±0.1	2±0.1
p2540-21	0	**90±0.4**	**45±0.2**	2±0	**2±0**	**2±0**
Neg. peptide	0	0	1±0.5	1±0	0	0
Ag+	**6±0.3**	**88±0.4**	**3±0.4**	2±0.1	2±0.1	2±0.2
Unstimulated PBMC	0	0	0	0	0	0

Significant positive % increase of memory $CD8^+$ T cells, IFN-γ producing T cells and % lysis are indicated in bold. The values are expressed as means ± SD. The results were calculated by subtracting the % of non-stimulated cells from peptide stimulated cells. (-) not determined.

4. DISCUSSION

Cytotoxic T cell responses are thought to play a significant role in the host defence against intracellular pathogens. Thus, the successful resolution of infection with *E. ruminantium* should involve the induction of CTLs that are capable of killing cells harbouring this pathogen. All previous studies have shown an increase in $CD8^+$ T cells but have not determined the cytotoxic activities induced by $CD8^+$ T cells after stimulation with the recombinant proteins [11]. A total of 14 *E. ruminantium* genes were cloned in pCMViUBs as part of a previous study [7] and were selected for this study. When these genes were tested in sheep as experimental DNA vaccines for *E. ruminantium*, only the 1H12 cocktail (Erum2540, -2550, -2580 and -2590), induced 100% protection. One reason for this may be that the unsuccessful proteins do not contain $CD8^+$ T cell epitopes. This may be due to the selection process followed in the past. The 1H12 antigens were cloned into a mammalian expression vector and tested in animals without any prior knowledge as to which immune response it activated [7, 8, 23]. Subsequent vaccine candidates were selected for their ability to produce IFN-γ in immune PBMC by using an IFN-γ ELISPOT assay and RT-qPCR to determine other cytokines induced [9, 10]. In these experiments, however, the cell types that produced the cytokines were unknown.

To test if any of the selected antigens, previously tested in animals, induce $CD8^+$ T cells to kill target cells, a human PBMC based FATT-CTL assay was adapted for use with sheep PBMC [21]. This assay was recently optimized for use with horse PBMC [22], and this study is the first to describe the optimization for use with sheep PBMC. Optimum lysis could be detected after 72 h incubation at an effector/target ratio of 1:1. The expression of GFP in the target cells was considered as an indicator that the assay is working and similar levels of GFP expression

were detected at all time points in PBMC from all the animals used. The lysis observed correlated significantly with increased $CD8^+$ T cells as determined by surface staining of effector cells.

Results presented in this study show that only five of the selected proteins (Erum0660, Erum2330, Erum2540, Erum2580 and Erum5000) induced cytotoxicity. The peptides of these were further analysed. Only Erum2540 protein was able to induce cytotoxicity in PBMC from all three immune sheep tested. Erum2540 is one of the ORFs in a cocktail of four 1H12 *E. ruminantium* ORFs (Erum2540, Erum2550, Erum2580 and Erum2590) which gave promising vaccine results [23, 7, 8]. However, significant CTL activities were detected for the remaining proteins in only one or two sheep whilst several of their peptides induced CTL in all three animals. For example, Erum2330 only induced CTL in s6821 while peptide p2330-1 induced CTL in all three sheep, and while Erum0660 induced CTL in PBMC from s6821, its peptides induced CTL in PBMC from s6822. Although the peptide induced responses varied between the animals, they appear to be less MHC restricted than the proteins. It would have been very interesting to determine the MHC I alleles for these animals. Several studies have indicated that MHC class I molecules are highly polymorphic and different alleles vary in their peptide binding specificity [24, 25, 26]. Fortunately, from a previous study it was determined that these three sheep share one MHC II (DRB1 second exon allele) and that the second allele is different [11]. This suggests that these three sheep are genetically different and that the variable CTL responses could be attributed to the different genetic makeup of these animals. This needs to be validated with peptide MHC binding assays. Of importance is that peptide Erum2540-6 was the only peptide that induced significant CTL, $CD8^+CD45RO^+$and $CD8^+$IFN-γ^+ by PBMC from all three sheep, and Erum2540 and p2540-20 induced the highest % CTL response in all three outbred sheep. Their ability to induce similar responses in a larger population will indicate their suitability for inclusion into a multi-epitope vaccine. Hence, confirmatory testing of the top candidates should be tested in a different and larger set of sheep in follow up experiments. Moreover, the peptides MHC class I specificity should be determined to ensure that those included in a vaccine are of different specificity. This will eliminate inhibition of binding of possible lower affinity peptides to MHC by higher affinity peptides [27]. Flow cytometry based assays can only determine whether T cells respond to a particular peptide/antigen but do not verify which MHC allele is involved. It would be necessary to perform additional research to determine to which allele a peptide is binding.

There were inconsistent CTL results between PBMC from animals tested with some protein and peptides. For example, Erum0660 showed significant CTL lysis with animal s6821 yet, none of the peptides derived from it induced CTL lysis. In contrast, CTL inducing Erum0660 protein and peptides were identified with s6823. It should be noted that not all peptides for this

protein were or could be tested. Only peptides that are conserved between 13 *E. ruminantium* strains were tested to ensure that they are able to protect against most strains. It is possible that the omitted peptides were better binders to the MHC class I alleles of s6821. On the other hand, Erum5000 peptides induced positive lysis in three sheep tested, yet no lysis was demonstrated using cells transfected with Erum5000 for animal s6823. This may indicate that there were inhibitory peptides present within the whole protein that were presented by MHC from s6823 and not by the other sheep.

In *silico* analysis was done for all the proteins tested using bovine MHC class I prediction algorithms since sheep MHC class I typing prediction algorithms do not exist. The analysis shows that for Erum2540 there is more than one predicted epitope derived from the peptide C terminal peptides. This may explain why there seems to be less MHC restriction when this protein is used. Previous studies have indicated that several patients recognised multiple epitopes from the *M. tuberculosis* ESAT-6 antigen [28]. These epitopes were scattered throughout the entire sequence of ESAT-6 and CMI was not restricted to only one or two immunodominant regions. Even though we found some correlation with prediction in Erum2540 and the experimental data, the bovine algorithms of the rest of the proteins predicted high to intermediate affinity binders across all five the proteins tested or the predictions did not correspond to those peptides that experimentally induced CTL. Perhaps using sheep specific MHC I in the algorithm will give a better correlation.

It should also be considered that the varied responses induced by the peptides in the three sheep might suggest that the frequency of these peptide specific CD8+ T cells was very low in the non-responder sheep and therefore, expansion of effector T cells was either delayed or not enough for lysis of the target T cells [29]. However, in this study, it was noted that in most cases high % lysis did not correlate with high % $CD8^+$ T cells and vice versa. It has been shown that some $CD8^+$ T cells are capable of expressing IFN-γ but exhibit little or no lysis activity [30, 31, 32]. Trautmann and co-workers [33] indicated that human $CD8^+$ T cells of the peripheral blood contain a low $CD8^+$ cytotoxic/effector subpopulation.

Of particular relevance to this study are peptides that induced CTL responses in all three sheep were highly conserved among 13 *E. ruminantium* strains (eg. p2540-6, p2540-8, p2540-12, p2540-18, p2540-20, and p2540-21). 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 [34, 35]. Genetic variation among *E. ruminantium* strains is one of the obstacles that hinders the progress in vaccine development [36]. 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 the potential to induce more effective recall T cell responses against different strains. Conserved regions of the bacterial genome are those that have been maintained through the evolution of the pathogen, therefore, variation would affect the function of the gene product [37, 38]. These results 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 [39, 40]. Furthermore, conserved epitopes of influenza A virus were shown to induce protective immunity mediated by influenza specific B- and T cells [41]. In addition, vaccine-induced human $CD8^+$ T cells through conserved subdominant Pol epitopes were able to control HIV-1 replication *in vitro* [42]. This knowledge complements our data that these epitopes may be useful in the design of a subunit vaccine.

Moreover, it cannot be excluded that other ORFs (Erum2550, Erum2580 and Erum2590) may have played a crucial role in protection in 1H12-inoculated sheep after needle challenge. Recently, the proteins encoding these ORFs were shown to induce proliferation of $CD4^+$ and $CD8^+$ T cells and IFN-γ production [43]. However, CTL-mediated immune responses were detected in only one sheep in all peptides selected except for p2580-12 that gave cytotoxic activities in all three sheep tested. The only possible reason could be that antigens predominantly induced $CD4^+$ T cells and to a lesser degree cytotoxic T cells ($CD8^+$) to proliferate when immune PBMC were stimulated.

In conclusion, the FATT-CTL assay was optimised for use in sheep PBMC. This study presents for the first time to our knowledge the identification of CTL epitopes of *E. ruminantium* peptides, in Erum2540, Erum2550, Erum2580, Erum2590, Erum0660, Erum2330 and Erum5000. These results correlate with similar studies where the role of cytotoxic T cells in immunity against several diseases induced by intracellular bacteria have been noted. This includes intracellular bacteria, *Chlamydia trachomatis* [44, 45, 46], *M. tuberculosis* [47, 48] and *Rickettsia conorii* [49]. CTL epitopes selected in this study can be incorporated into the vaccine design with the goal of inducing broadly reactive responses. In addition, combining epitopes from different antigens with T-helper and CTL activity will induce a broader spectrum of immune responses than a DNA vaccine encoding a single antigen. Hence, peptides that induce CD8 and $CD4^+$ T cells should be included in the final vaccine formulation. Naturally, pathogens do not display a single antigen to the immune system, multiple copies and different antigens are presented to the system. The advantages of these vaccines therefore include, better coverage of natural pathogen antigen diversity, better match the genetic variability of the target population immune system, reduce the risk of pathogen escape due to immune pressure and may even target several life stages of pathogens [34]. Our results show that

these peptides could serve as promising candidates for a multi-epitope vaccine against *E. ruminantium*.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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