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Citation for published version:

Morrison, I, Aguado-Martinez, A, Sheldrake, T, Palmateer, NC, Ifeonu, OO, Tretina, K, Parsons, K, Fenoy, E, Connelley, T, Nielsen, M & Silva, JC 2021, 'CD4 T cell responses to *Theileria parva* in immune cattle recognise a diverse set of parasite antigens presented on the surface of infected lymphoblasts', *The Journal of Immunology*, vol. 207, no. 8, pp. 1965-1977. <https://doi.org/10.4049/jimmunol.2100331>

Digital Object Identifier (DOI):

[10.4049/jimmunol.2100331](https://doi.org/10.4049/jimmunol.2100331)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

The Journal of Immunology

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1 CD4 T cell responses to *Theileria parva* in immune cattle recognise a diverse set of parasite
2 antigens presented on the surface of infected lymphoblasts

3

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26 **Keywords:** *Theileria parva*, cattle, vaccination, CD4 T cell, antigens, polymorphism

27

28 **Running Title:** Specificity of CD4 T cell responses to *Theileria parva*

29

30 This work was supported by a grant awarded by the Bill and Melinda Gates Foundation
31 jointly with the UK Department for International development (DfID) [No. OPP1078791] and
32 a Biotechnology and Biological Sciences Research Council (BBSRC) Institute ISP grant
33 (grant number BB/J004227/1).

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60 **Abstract**

61 Parasite-specific CD8 T cell responses play a key role in mediating immunity against
62 *Theileria parva* in cattle (*Bos taurus*) and there is evidence that efficient induction of these
63 responses requires CD4 T cell responses. However, information on the antigenic specificity
64 of the CD4 T cell response is lacking. The current study used a high-throughput system for
65 antigen identification using CD4 T cells from immune animals to screen a library of ~40,000
66 synthetic peptides representing 499 *T. parva* gene products. Use of CD4 T cells from 12
67 immune cattle, representing 12 class II MHC types, identified 26 antigens. Unlike CD8 T cell
68 responses, which are focused on a few dominant antigens, multiple antigens were
69 recognised by CD4 T cell responses of individual animals. The antigens had diverse
70 properties, but included proteins encoded by two multi-member gene families – five haloacid
71 dehalogenases and five subtelomere-encoded variable secreted proteins (SVSPs). Most
72 antigens had predicted signal peptides and/or were encoded by abundantly transcribed
73 genes, but neither parameter on their own was reliable for predicting antigenicity. Mapping of
74 the epitopes confirmed presentation by DR or DQ class II alleles and comparison of
75 available *T. parva* genome sequences demonstrated that they included both conserved and
76 polymorphic epitopes. Immunisation of animals with vaccine vectors expressing two of the
77 antigens demonstrated induction of CD4 T cell responses capable of recognising parasitised
78 cells. The results of this study provide detailed insight into the CD4 T cell responses induced
79 by *T. parva*, and identify antigens suitable for use in vaccine development.

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81 **Key points**

- 82 • Multiple CD4 T cell antigens identified by screening a *T. parva* peptide library
- 83 • Antigens have diverse properties and include polymorphic and conserved proteins
- 84 • Parasite infection and viral-vector delivered antigen induce similar CD4 responses

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87 **Introduction**

88 Intracellular protozoan parasites induce T cell-mediated immune responses that play a
89 central role in development of immunity. However, the antigenic complexity of these
90 parasites presents a significant challenge for identifying the antigens that mediate immunity
91 and hence are candidates for vaccine development. One such pathogen is the tick-borne
92 parasite *Theileria parva*, which causes one of the most economically important diseases
93 affecting cattle in sub-Saharan Africa. The acute and frequently fatal nature of the disease in
94 cattle results in high levels of mortality and presents major challenges for implementing
95 control measures (1). Control relies predominantly on prevention of infestation with the tick
96 vector and in some regions use of vaccination. The latter involves an infection and treatment
97 regime using live parasites harvested from ticks and cryopreserved prior to use,
98 administered along with a long-acting formulation of oxytetracycline (2). The most frequently
99 used version of this vaccine incorporates three parasite isolates, to overcome parasite
100 antigenic diversity (3), two of which have recently been shown to be nearly identical
101 genome-wide (4). Although shown to be effective in the field (5), widespread application of
102 this multi-component live vaccine has been hampered by the cumbersome processes
103 involved in its production and quality control and the requirement of a cold chain for vaccine
104 distribution. In some regions, concerns about potential risks of introducing the vaccine
105 parasite strains into local tick populations have also resulted in reluctance to adopt
106 vaccination. These shortcomings have led to efforts to develop alternative vaccines based
107 on the use of defined parasite antigens (reviewed in 6).

108 Strategies adopted for development of subunit vaccines have built on knowledge of immune
109 responses in animals immunised with live parasites. A large body of evidence indicates that
110 immunity against *T. parva* is mediated by T cell responses against the intra-lymphocytic
111 schizont stage of the parasite and that parasite-specific CD8 T cells play a central role in
112 immunity (reviewed in 7). Notably, it has been possible to confer immunity against parasite
113 challenge by adoptive transfer of purified actively responding CD8 T cells from immune to

114 naïve identical twin calves (8). Moreover, CD8 T cell responses in cattle immunised with a
115 single parasite isolate are frequently strain-restricted, which has been shown to correlate
116 with susceptibility to subsequent challenge with heterologous parasite strains (9). A striking
117 feature of the parasite-specific CD8 T cell response is that the response in individual animals
118 is focused on a few highly dominant antigens, which are often polymorphic (10). This
119 immunodominance is critical in determining the strain restriction of immunity.

120 More recent work has successfully identified a series of *T. parva* antigens recognised by
121 CD8 T cells from immune cattle (11, 12). However, although immunisation of cattle with
122 some of these antigens, employing prime-boost protocols with recombinant viral vectors,
123 successfully induced parasite-specific CD8 T cell responses, protection against parasite
124 challenge was only achieved in a proportion of the immunised animals (11, 13).

125 The adoptive transfer studies referred to above utilised CD8 T cells collected during the
126 active response of immune calves to challenge with *T. parva*. Hence, it remains unclear
127 whether or not other components of the immune response are required for efficient activation
128 of memory CD8 T cells following parasite challenge. Help from CD4 T cells is required for
129 efficient induction of CD8 T cell responses against many viral infections (Reviewed in 14).
130 Moreover, studies of responses to *Plasmodium* parasites using murine models have shown
131 that the induction and maintenance of CD8 T cell responses to the pre-erythrocytic stage of
132 the parasite are strongly dependent on the presence of specific CD4 T cells (15-17). *T.*
133 *parva*-infected lymphoblasts express class II MHC on their surface and immune animals
134 exhibit strong parasite-specific CD4 T cell responses against parasitized cells (7, 18).

135 However, their role in immunity has received relatively little attention. Bovine CD8 T cells do
136 not appear to produce IL-2 upon activation and *in vitro* studies have indicated that their
137 ability to respond following antigen recognition is dependent on production of soluble growth
138 factors by other cell types. Experiments using different combinations CD4 and CD8 T cells
139 purified from genetically identical *T. parva*-immune or -naïve twin calves have demonstrated
140 that optimal proliferation of the immune CD8 T cells required the presence of antigen-

141 specific CD4 T cells (19). Moreover, the results indicated that both cell types had to
142 recognise antigen presented by the same *T. parva*-infected cells. These observations
143 suggest that successful vaccination against *T. parva* with defined antigens requires induction
144 of both CD4 and CD8 T cell responses of appropriate specificities and functional activities.
145 Although a series of early studies identified crude antigenic fractions of parasitized cells
146 recognised by *T. parva*-specific CD4 T cells (18, 20-22), the precise antigenic specificities of
147 the responding T cells have not been defined.

148 Since the development of efficacious vaccines against *T. parva* is likely to require inclusion
149 of CD4 as well as CD8 T cell antigens to provide robust protection, the current study set out
150 to identify the parasite antigens recognised by CD4 T cell responses in cattle immunised
151 against *T. parva* by infection and treatment. Antigens were identified by undertaking a high-
152 throughput screen with CD4 T cells from immune cattle of diverse MHC types, using a library
153 of peptides representing 499 parasite proteins (approximately 12.5 % of the parasite
154 proteome). The results demonstrate that, in contrast to the narrow specificity of CD8 T cell
155 responses, CD4 T cells from immune cattle recognise multiple antigens presented by
156 parasite-infected cells and that such responses can readily be induced using viral vaccine
157 vectors.

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166 **Methods**

167 **Animals and immunisation**

168 The study utilised castrated male Holstein cattle between 12 and 24 months of age. All
169 animals were selected by initially determining their MHC class I types using a combination of
170 serological typing with monoclonal antibodies (23) and allele-specific PCR (24). Some of the
171 animals, including two DR 011.01-homozygous animals, produced by father-daughter mating
172 with sires of known genotype, were included. The class II DRB3 alleles expressed by the
173 selected animals were identified by sequencing of cloned PCR products obtained from cDNA
174 with DRB3-specific primers (25).

175 Animals used for antigen screening were immunised with the Muguga isolate of *T. parva* by
176 infection with cryopreserved sporozoites and simultaneous treatment with a slow-release
177 formulation of oxytetracycline, as described previously (2). Additional animals were
178 immunised with selected antigens expressed individually in recombinant human adenovirus
179 (hAd5) and vaccinia virus (modified Ankara strain - MVA) vectors. Animals received 2×10^9
180 IU of each adenovirus followed 8 weeks later by 5×10^8 PFU of each vaccinia virus, both
181 administered intramuscularly.

182 **T cell lines**

183 All cultures of parasitized cells and T cells were conducted in RPMI 1640 medium
184 supplemented with 10% foetal bovine serum, 2-mM L-glutamine, 5×10^{-5} M 2-mercapto-
185 ethanol and penicillin and streptomycin. Cell lines infected with the Muguga stock of *T.*
186 *parva*, from which the reference genome sequence was derived (26), were generated by
187 infection of peripheral blood mononuclear cells (PBMC) *in vitro* with sporozoites (27). A
188 parasite-specific CD4 T cell line was generated from each immune animal as described
189 previously (28); briefly, PBMC were stimulated with irradiated autologous *T. parva*-infected
190 cells three times at weekly intervals, followed by depletion of CD8 and $\gamma\delta$ T cells by
191 antibody-and-complement-mediated lysis. In some cases, the cultures were subjected to a

192 further positive selection step involving cell sorting of cells stained with a CD4-specific
193 monoclonal antibody (IL-A11) using a FACSaria cell sorter (Becton-Dickenson Biosciences,
194 Oxford, UK). Purified CD4 T cells (>98%) were maintained by stimulation at 7-10 day
195 intervals with equal numbers of irradiated *T. parva*-infected cells in medium containing 50
196 units/ml of recombinant human IL-2 (Chiron, Emryville, CA, USA)..

197 **Antigen screening**

198 A library of synthetic peptides representing 499 selected *T. parva* gene products was used
199 for antigen screening. The gene products included in the library are listed in Supplementary
200 Table S1. This library consisted of 40,921 18-mer peptides overlapping by 12 residues,
201 produced as a series of 819 pools each containing 50 contiguous peptides. Peptides were
202 supplied by JPT Peptide Technologies GmbH (Berlin, Germany) and were produced by their
203 Microscale system, which results in an additional glycine residue at the C-terminus of each
204 peptide. Antigen screening was conducted in 96-well round-bottomed plates by incubation of
205 each peptide pool with 2×10^4 CD4 T cells/well in a total volume of 150 μ l of medium, each
206 peptide at a final concentration of 2 μ g/ml. CD4 T cells harvested 9-10 days after antigenic
207 stimulation were used for antigen screening to ensure low background levels of cytokine
208 secretion. Because of the large numbers of CD4 T cells required for each screen, they were
209 conducted in single wells. After incubation with peptide at 37°C for 48 h, culture
210 supernatants collected from the wells were assayed for IFN γ , using a biological assay
211 performed as described previously (29), based on the ability of the IFN γ in culture
212 supernatants to up-regulate surface expression of MHC class II on Madin Darby Bovine
213 Kidney (MDBK) cells. The MDBK cells were harvested after 48 hours and class II MHC
214 expression assessed by flow cytometry following immunofluorescence staining with a class II
215 DR-specific monoclonal antibody (IL-A21). Results are expressed as the percentage of
216 MDBK cells expressing surface class II.

217 For each T cell line, the screening assay was repeated with a limited set of peptide pools
218 (Supplementary Table S2) comprising all pools that were positive in the initial screen, other

219 pools that contained peptides from antigens represented in the positive pools and an
220 additional 15 randomly selected pools that gave negative results with all T cell lines. This
221 secondary screen, as well as all subsequent assays of the positive antigens were conducted
222 in duplicate wells. Only supernatants that resulted in at least 20 percentage points above the
223 background levels of class II expression in both the primary and secondary screens were
224 considered positive (all were statistically significant at $p < 0.01$ in the secondary screen). The
225 50 peptides in each pool sometimes originated from a single gene product, but in other
226 cases were derived from two or occasionally three gene products. In the latter cases,
227 subcomponents of the pools were re-screened in duplicate to determine which gene product
228 gave rise to the positive response.

229 **Epitope localisation and MHC restriction**

230 For each positive peptide pool, the epitope regions within the parasite protein recognised by
231 CD4 T cells were identified by repeating the screens with individual 18-mer peptides
232 (overlapping by 12 residues).

233 Two assays were used to determine whether peptides were presented by class II DR or DQ
234 MHC proteins: A first assay used Chinese hamster ovary (CHO) cells expressing either of
235 two defined bovine DR alleles, namely DR 010:01 and DR 011:01, known to be expressed
236 by MHC-homozygous animals used in the study. These cells were produced by transfecting
237 CHO cells with a construct containing cDNAs encoding both the DR α and β chains linked by
238 an oligonucleotide from foot and mouth disease virus, which encodes a self-cleaving peptide
239 (30). Cells transfected with this construct were stained with Mab IL-A21 and positive cells
240 purified by cell-sorting were expanded and, after checking for DR expression, aliquots of the
241 cells were cryopreserved prior to use. For the T cell assay, resuscitated CHO cells were
242 distributed in 96-well u-bottomed plates at 10^4 /well and duplicate wells were incubated with a
243 range of concentrations of peptide (2 μ g to 100ng/ml) or medium for two hours. The cells
244 were then washed twice with culture medium and T cells added to the wells at 10^4 /well. After

245 incubation at 37°C for 48 hours, supernatants were harvested and assayed for IFN γ using
246 the biological assay.

247 A second approach involved repeating the screening assay as described above with the
248 individual positive peptides, with or without addition of monoclonal antibodies specific for
249 bovine DR (Mab IL-A21) or DQ (Mab CC158). Because of the use of class II-specific
250 antibodies in these experiments, IFN γ release could not be measured using the biological
251 assay and therefore was measured using a sandwich ELISA as described previously (31).

252 **Properties of gene products**

253 Antigenic proteins need to have access to MHC-processing pathways in the host cell. As
254 such, we identified genes whose products are targeted to the secretory pathway or are
255 otherwise associated with membranes. TargetP 1.1 (32, 33) was used to identify proteins
256 predicted to be targeted to the secretory pathway with high reliability (reliability classes 1).
257 Proteins were predicted to be glycosylphosphatidylinositol (GPI)-anchored using GPI-SOM
258 (34) and PredGPI (35). The presence of five or more transmembrane helices, which is a
259 strong indicator of a transmembrane protein, was determined with TMHMM (36, 37).
260 Selected proteins were all produced from genes transcribed in the schizont stage of the
261 parasite and transcript abundance was assessed from the single published dataset on the
262 transcriptome of *T. parva* (38).

263 **Polymorphism**

264 For each locus, the orthologous allele was obtained from the draft genome assembly of 17
265 *T. parva* strains isolated from cattle from across the range of the distribution of the parasite.
266 The genome sequence of each strain was generated as described before (39). When alleles
267 were mostly or completely missing from the draft assembly, the strain was removed from the
268 respective dataset. When only a few nucleotides were missing in the 3' or 5' end, the
269 sequence was kept in the dataset and the missing nucleotides were assumed to be identical
270 to the haplotype that was otherwise identical to it. The nucleotide sequences were aligned

271 using Muscle (40) and manually curated in Mesquite (41). Nucleotide diversity per site
272 averaged across each gene was estimated for silent sites (synonymous; average number of
273 synonymous differences per synonymous site, π_S) and amino acid-changing sites (non-
274 synonymous; average number of non-synonymous differences per non-synonymous site,
275 π_{NS}), using DnaSP (42). In addition, π_{NS} was estimated across sliding windows of non-
276 synonymous sites, with width and step, respectively, of 30 and 5 non-synonymous sites.
277 Regions of low complexity and micro- and mini-satellites were identified using Tandem
278 Repeats Finder (43). Plots of nucleotide diversity were drawn using R (44).

279 **Statistical analysis**

280 Data are presented as means and standard deviations (SD). Statistical analyses were
281 performed using Student's T test. Statistically significant differences compared with the
282 control are indicated by asterisks - p values of $<0.05 = *$ and $<0.01 = **$.

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294 **Results**

295 **Selection of parasite proteins for generating the peptide library**

296 The approach used for selection of the parasite gene products to produce the peptide library
297 for antigen screening is illustrated in Figure 1 and the content of the library is listed in
298 Supplementary Table S1. Selection of proteins was based predominantly on the possession
299 of a predicted signal peptide, indicating likely secretion from the intracellular parasite (45),
300 and/or high abundance of transcription of the genes in infected lymphoblasts. Ten proteins
301 previously shown to be CD8 T cell antigens were included (11, 12). A re-annotated version
302 of the *T. parva* genome was used to produce the parasite proteome sequence (46), from
303 which 420 proteins highly likely targeted for the secretory pathway were selected; 39 of
304 these have a likely GPI anchor and 47 have five or more transmembrane helices, with some
305 overlap among those sets (Fig 1). An additional 69 proteins were selected based on
306 abundance of transcription. .

307 **Screening of the peptide library**

308 A *T. parva*-specific CD4 T cell line from each of the 12 immunised cattle was used for
309 antigen screening. The donor cattle represented 12 different class II DRB3 alleles and
310 included animals that were homozygous for 5 different MHC haplotypes (Table 1). Pairs of
311 cattle homozygous for DR 010.01 and DR 011.01 had been produced by father-daughter
312 mating and although they were not typed for class II DQ are also highly likely to share the
313 same DQ alleles. Screening of the peptide library (819 peptide pools representing 499
314 parasite proteins) with these CD4 T cell lines resulted in the identification of positive peptide
315 pools in all animals; 32 of the pools yielded positive results. A secondary screen of all
316 positive pools was used to confirm positive results and further screens of the sub-
317 components of pools that contained peptides from more than one parasite protein were
318 carried out. The results identified a total of 26 positive parasite proteins, representing 5.2%
319 of the gene products screened. Representative results obtained with five CD4 T cell lines in
320 the secondary screen (pools listed in Supplementary Table S2) are shown in Figure 2 and a

321 summary of the gene products recognised by each animal is shown in Table 1. CD4 T cell
322 lines from individual animals recognised between 2 and 10 antigens (mean of 6.2). There
323 was considerable variation between animals in the antigenic specificity of the detectable
324 response. None of the antigens were recognised by all animals; some were detected by only
325 one or two T cell lines, whereas responses to others were detected in up to 8 of the 12 lines
326 assayed. Within the cell lines tested, two pairs of animals homozygous for DR 010:01 and
327 DR 011:01 each shared responses to 5 antigens ($5/11 = 45.5\%$ and $5/8 = 62.5\%$ of the
328 antigens identified respectively), which tended to be the strongest responses detected in
329 these animals, confirming that specificity was influenced by MHC type. Differences in
330 detection of responses to some antigens by the same DR genotypes may relate to
331 differences in the overall antigenic specificity of the T cell lines, resulting in failure to detect
332 some weak specificities, and/or differences in the T cell receptor repertoire of the animals.

333 **Characteristics of the target antigens**

334 As shown in Table 2, which summarises the main features of the antigens, they are encoded
335 by genes distributed across all four chromosomes and exhibit a range of properties. They
336 include six gene products known to induce CD8 T cell responses, four of which (Tp1, Tp2,
337 Tp7 and Tp9) were reported previously (11, 12) and the remaining two (Tp32 and Tp33)
338 identified in recent antigen screens (N. D. MacHugh and W. I. Morrison unpublished data).
339 Several members of two multi-member gene families, namely the haloacid dehalogenases
340 (HAD) and the subtelomere-encoded variable secreted proteins (SVSP), are represented in
341 the antigen panel. All five HAD proteins and five of the 14 SVSP proteins, included in the
342 peptide library, were identified as antigens.

343 The panel of gene products included in the peptide library used for antigen screening was
344 biased towards abundantly expressed genes and proteins predicted to be secreted from the
345 parasite into the host cell cytoplasm or be otherwise exposed to the host. Reflecting this
346 bias, a majority of the antigens recognised by CD4 T cells (19/26) have a predicted signal
347 peptide; however, seven antigens lacked a signal peptide. Only three of the antigens

348 predicted to be secreted (Tp18, Tp19 and Tp36) showed evidence of a membrane anchor –
349 the former having a predicted transmembrane domain and the latter two a predicted GPI
350 anchor. Biological studies have confirmed that these three proteins (previously referred to as
351 PIM, p104 and gp34) are localised to the schizont surface (47-49). Tp19 (p104) and Tp36
352 (gp34), as well as the *T. annulata* orthologue of another protein (Tp33), have been found to
353 be within a complex of host and parasite proteins that associate with host microtubules on
354 the surface of the schizont (50). The abundance of transcription of the antigen-encoding
355 genes showed wide variation. While some of the antigens ranked very highly in their levels
356 of transcription, including those selected on the basis of transcript abundance, others fell
357 within the 20% genes with lowest levels of expression. In summary, no single parameter
358 could be used reliably to predict antigenicity.

359 **Epitope identification and MHC restriction**

360 Experiments to identify the target epitopes were undertaken to provide reference information
361 on the fine specificity of the response, with which to compare vaccine-induced responses.
362 This work focused on three DR types (010:01, 011:01 and 016:01), for which the CD4 lines
363 were derived from class II-homozygous animals. These included pairs of animals
364 homozygous for DR 010:01 and DR 011:01. Examples of results obtained by screening 5 of
365 the peptide pools are shown in Figure 3. The five CD4 T cell lines examined recognised 21
366 of the antigens identified. Screening of individual peptides for 19 of these antigens identified
367 one or occasionally two or three epitope regions within each positive pool. Where reactions
368 to two contiguous (18-mer) peptides were detected, testing of truncated peptides allowed
369 each epitope to be localised to within a region of 18 amino acids or less. The results of these
370 assays, summarised in Table 3, resulted in identification of 11-14 epitopes for each of the 3
371 MHC types examined. However, the HAD antigens recognised by animal 2824 contained an
372 epitope that showed a high level of identity between all five antigens; the epitope sequences
373 in Tp17 and Tp22 were identical and the sequences of the other three (Tp14, Tp21 and
374 Tp22) differed at one, six and three of the 15 amino acid residues respectively (Table 3). The

375 same epitopes were recognised by animal 598 (data not shown), which also expresses the
376 DR 016:01 allele. Since four of these proteins were only recognised by these two DR
377 016:01-positive animals, it was not possible to discern whether all or only some of the
378 antigens were responsible for inducing the CD4 T cell responses.

379 As expected, MHC type influenced the epitopes that were recognised. First, where antigens
380 were recognised by animals of identical MHC types (DR 010:01 or DR 011:01), the CD4 T
381 cell lines recognised the same epitope; conversely, within the five antigens recognised by
382 two or three of the DRB3 types (Tp16, Tp9, Tp20, Tp18 and Tp15) the majority of the
383 epitopes recognised (13/17) were unique to a single DRB3 type. However, in four instances
384 the same peptide was recognised by animals with two different class II types (Tp9₂₈₀₋₂₉₅ by
385 DR 011:01 and DR 016:01; Tp9₂₉₂₋₃₁₀ by DR 010:01 and DR 016:01; Tp18₉₇₋₁₁₄ by DR
386 010:01 and DR 011:01; Tp20₁₀₃₋₁₂₀ by DR 010:01 and DR 016:01).

387 The results obtained by testing the ability of CHO cells transfected with the DR 010:01 or DR
388 011:01 class II MHC alleles to present peptides identified on the respective MHC
389 backgrounds are shown in Figure 4 and summarised in Table 3. Thirteen of the 22 identified
390 epitope specificities (seven on the DR 010:01 and six on the DR 011:01 backgrounds) were
391 recognised when presented by CHO cells expressing the relevant DR allele. The results
392 obtained by testing the ability of DR- and DQ-specific monoclonal antibodies to inhibit
393 epitope recognition confirmed that those epitopes that were not recognised on DR-
394 transfected CHO cells were inhibited by the DQ-specific antibody but not by the DR-specific
395 antibody (Figure 5). Epitopes identified on the DR 016:01 background were tested only
396 using the latter assay; recognition of ten of the eleven epitopes tested was inhibited by the
397 DR-specific antibody but not by the DQ-specific antibody. The converse result was obtained
398 with the remaining epitope (Data not shown).

399 Hence, overall twenty-two of the unique epitopes analysed were found to be DR-restricted
400 and eight were DQ-restricted. Of the four epitopes that were recognised by two different
401 class II types, two (Tp9₂₈₀₋₂₉₅ and Tp20₁₀₃₋₁₂₀) were found to be DR-restricted and two (Tp9₂₉₂₋

402 ₃₁₀ and Tp18₉₇₋₁₁₄) DQ-restricted. In separate studies, the two DR-restricted epitopes were
403 found to have a high predictive score for binding to both DR alleles (M. Nielsen, unpublished
404 data).

405 **Polymorphism of the identified antigens**

406 The identification of epitopes recognised by the CD4 T cell response in individual animals,
407 coupled with recently generated genome sequences from 17 parasite isolates obtained from
408 cattle, allowed us to estimate the level of polymorphism across the length of the target
409 antigens, including regions encoding the CD4 epitopes. The target antigens ranged from
410 some of the most polymorphic in the *T. parva* genome (39), such as Tp2 and Tp9, to those
411 that are completely conserved (Table 2). They vary greatly in length and in the presence of
412 low complexity regions or repeat regions (Figure 6). Some of the identified epitopes were
413 located in antigen regions with intermediate to high variability in the parasite population (e.g.
414 Tp2, Tp9, Tp15 – Figure 6), whereas others were in conserved regions of antigens (e.g. Tp1,
415 Tp15, Tp16, Tp30 – Figure 6). In a few cases, epitopes were found in regions with length
416 variation (e.g. Tp9 – Figure 6), such that they are absent in some strains, or those adjacent
417 to repeats (e.g. Tp9, Tp15 – Figure 6), suggesting that protein conformation nearby may
418 alter access to the epitopes. Overall, the CD4 T cell responses of all animals were found to
419 recognise both conserved and polymorphic epitopes.

420 **Specificity of CD4 T cell responses induced by prime-boost immunisation with** 421 **selected antigens**

422 The context in which antigens are delivered can sometimes alter the selection of epitopes
423 (51), with obvious consequences for the efficacy of vaccination. To determine whether *T.*
424 *parva* antigens delivered in vaccine vectors induce CD4 T cell responses of similar
425 specificities to those elicited by live parasites, four animals expressing DRB3 010:01 and/or
426 DRB3 011:01 were immunised using a prime-boost protocol, with the Tp9 and Tp15 antigens
427 expressed in recombinant adenovirus (Ad) and vaccinia (MVA) vectors. CD4 T cell lines
428 generated from all four animals, by stimulation of T cells with autologous *T. parva*-infected

429 cells, recognised Tp9 and Tp15 (Table 4). Eight epitopes had been identified in these two
430 antigens during antigen screening with T cell lines expressing DRB3 010:01 or DRB3
431 011:01. Epitope mapping of the CD4 response in one of the viral vector-immunised animals,
432 which expressed both DRB3 010:01 and DRB3 011:01, revealed significant responses of
433 variable magnitude to 6 epitopes, four in Tp9 and two in Tp15. These epitopes were identical
434 to those recognised by *T. parva*-immune animals (Table 5), confirming the capacity of the
435 vectored antigens to induce CD4 T cell responses to epitope specificities displayed on
436 infected cells.

437 The same four animals were concurrently immunised with recombinant Ad/MVA viruses
438 expressing a third antigen (Tp10) (12) known to induce CD8 T cell responses but not
439 identified as a CD4 T cell target antigen by screening in the current study. All 4 four animals
440 generated a CD4 T cell response to Tp10, detectable by stimulation *in vitro* with *T. parva*-
441 infected cells (Table 4). Hence, this antigen is presented by parasitised cells and can be
442 immunogenic (ie. outwith the context of natural infection), even though it was not identified
443 as a CD4 T cell antigen in the peptide screens.

444 Data on CD8 responses to these antigens were also obtained from 3 of the animals.
445 Significant responses were detected in all 3 animals for Tp9 and in two animals for Tp10, but
446 none of the animals responded to Tp15 (Table 4).

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454 Discussion

455 The evidence that CD8 T cells play a key role in immunity to *T. parva* has led to efforts to
456 develop a vaccine using CD8 T cell target antigens. CD4 T cell responses are also likely to
457 be required to provide help for efficient induction and recall of CD8 T cell responses.

458 Although infection with *T. parva* induces vigorous CD4 T cell responses against infected
459 cells, there is a paucity of information on their specificity. The results of the current study
460 demonstrate that, in contrast to the profound immunodominance of CD8 T cell responses to
461 *T. parva* in immune cattle, whereby a large proportion of the response in individual animals
462 is focused on one or two antigens (10), CD4 T cell responses recognise multiple antigens,
463 including both polymorphic and conserved antigens. Antigen screens using CD4 T cells from
464 twelve immune cattle of different MHC class II types identified 26 antigens, with up to eleven
465 antigenic specificities detected in a single animal. As would be expected, there was variation
466 between animals in the antigens that were recognised, with no single antigenic specificity
467 detected in all animals.

468 Despite the multiple antigens identified in this study, the findings almost certainly
469 underestimate the diversity of antigenic specificities presented by *T. parva*-infected cells.
470 Since the peptide library represented only about 12.5% of *T. parva* genes, almost all of
471 which are transcribed by the schizont stage of the parasite (46), the remaining proteome is
472 likely to include additional unidentified antigens. Moreover, the assay used for antigen
473 screening may well fail to detect T cell specificities present at low frequency. The finding that
474 immunisation of calves with viral vectors expressing the Tp10 CD8 antigen, which was not
475 detected by the antigen screens, induced CD4 responses reactive with *T. parva*-infected
476 cells, clearly illustrates the presence of additional immunogenic antigenic specificities on
477 infected cells.

478 The strategy employed in the current study for antigen screening was based on knowledge
479 of the biology of the parasite and how parasite antigens are likely to be recognised by CD4 T
480 cells *in vivo*. Unlike many other apicomplexan parasites, *T. parva* is found free within the

481 host cell cytoplasm, having escaped from the endocytic vacuole shortly after initial cell
482 invasion by the sporozoite (52). The parasite establishes an intimate relationship with the
483 host cell, which results in activation and transformation of infected host cells and inhibition of
484 apoptosis (53). Synchronous division of the parasite and host cell (54, 55) results in clonal
485 expansion of the infected cell population, thus ensuring that the parasites are retained
486 intracellularly throughout this stage of development. The transformed state of the infected
487 cells also results in expression of surface DR and DQ class II MHC proteins, as well as co-
488 stimulatory proteins such as CD80, CD86 and CD40 (W I Morrison, unpublished data).
489 These properties of infected cells favour endogenous routes of processing of parasite
490 antigens and allow direct recognition of infected cells by parasite-specific CD4 T cells, in the
491 absence of added antigen-presenting cells. Hence, the current study used CD4 T cell lines
492 generated from immune cattle by stimulation *in vitro* with intact parasitized cells for antigen
493 screening. Holstein cattle, which are highly susceptible to *T. parva* and are extensively used
494 for milk production in endemic areas of Africa, were used in the study. The class II MHC
495 types of the animals included the most frequently expressed DR alleles in Holsteins; a recent
496 study of 331 Holstein cattle identified 15 DR alleles, 10 of which were represented in the
497 current study and included the 7 most frequently detected alleles (56).

498 *T. parva* schizont antigens targeted by CD4 T cells on infected cells must access the
499 cytoplasm of the infected cells, prior to entering class II MHC processing pathways.
500 Consequently, proteins secreted from the parasite or released from the parasite by other
501 mechanisms represent likely candidate antigens. Studies of antigen processing of a number
502 of human and murine intracellular pathogens indicate that cytoplasmic proteins can be
503 transferred into the endosomal pathway for class II processing either following autophagy
504 within the cytoplasm or as peptides generated by degradation of parasite proteins within the
505 proteasome (57, 58). The involvement of the latter pathway has been demonstrated for
506 some CD4 T cell responses to influenza virus-infected cells (59-61). In preliminary studies,
507 we have found that recognition of the Tp9 antigen on infected cells by specific CD4 T cell

508 clones is inhibited by pre-incubation of the infected cells with the proteasome inhibitor
509 Epoxomicin (A. Aguada and W. I. Morrison, unpublished data), suggesting that this
510 endogenous pathway is involved in processing *T. parva* antigens.

511 A high-throughput antigen screen using a peptide library representing 499 parasite proteins
512 was used for antigen identification. As discussed above, given the requirement of antigens to
513 access the host cell cytoplasm, selection of parasite proteins for generating the peptides was
514 biased, firstly towards proteins predicted to have a signal peptide and therefore likely to be
515 secreted from the parasite and secondly towards genes that are abundantly transcribed in
516 the schizont stage of the parasite and whose products may access the cytosol by other
517 undefined routes. The selected parasite proteins also included those known to elicit antibody
518 or CD8 T cell responses against this stage of the parasite in immune cattle (11, 47, 62, 63).

519 Only 6 of the 14 CD8 antigens identified to date (Tp1, Tp2, Tp7, Tp9, Tp32 and Tp33), were
520 also identified as CD4 T cell antigens. Previous studies have shown that Tp1 and Tp2 are
521 highly dominant CD8 antigens in class I A18 and A10 animals respectively, yet these
522 antigens were not recognised, to a detectable level, by CD4 T cells from animals of these
523 class I genotypes. Thus, the CD4 and CD8 T cell responses in these animals have different
524 dominant antigenic specificities. A recent study of prime-boost immunisation of class I A18+
525 animals with Tp1 expressed in adeno and vaccinia virus vectors observed Tp1-specific CD4
526 responses during the active response to the vaccinia boost. However, since the T cell
527 responses were monitored using Tp1 antigen added to antigen-presenting cells, the findings
528 did not confirm recognition of the antigen presented by infected cells. Nevertheless, the
529 findings suggest that, as with our results with vector-induced responses to Tp10, vaccination
530 with defined antigens may elicit specificities that are not readily detected during responses to
531 infection.

532 All three proteins previously shown to induce antibody responses to the schizont stage of the
533 parasite in infected animals (PIM, p104 and p150 – now respectively, Tp18, Tp19 and Tp20)
534 (47, 62, 63) were identified as CD4 antigens, but only in a subset of the animals. Notably,

535 CD4 T cell responses to the Tp18 antigen (PIM), which is an abundant schizont protein and
536 induces highly dominant antibody responses in all immune animals (47), were detected in
537 only two of the animals examined. This implies that additional exogenous routes of
538 processing of some antigens released from infected cells may be involved *in vivo* in
539 generating CD4 T cell responses, due to more efficient display of antigen on antigen-
540 presenting cells. Although a majority of the identified antigens (19/26) had predicted signal
541 peptides, this appeared to reflect the composition of the peptide library, 84% of which was
542 generated from proteins with a predicted peptide sequence. Abundance of transcription of
543 the antigen-encoding genes was highly variable; while half of the antigens (13/26) were
544 encoded by the top 10% most abundantly transcribed genes, others were in the lower 50%.
545 Because transcript abundance was used to select proteins that lacked a signal sequence,
546 the antigens identified within this subset were encoded by some of the most abundantly
547 transcribed genes (10/11 ranked in the top 100). Although neither of these parameters on
548 their own appeared reliable for predicting CD4 cell target antigens, the large number of
549 antigens detected in the current study suggest that both properties may have been useful for
550 antigen identification. However, screening of a large unbiased panel of parasite gene
551 products would be required to address this question.

552 While the identified CD4 antigens included proteins with diverse properties, several
553 members of the haloacid dehalogenase (HAD) and the subtelomere-encoded variable
554 secreted protein (SVSP) families of proteins were identified. All five HAD proteins included in
555 the peptide library were identified as antigens. However, epitope mapping revealed a CD4 T
556 cell epitope that was highly conserved between the five HAD proteins and, since this was the
557 only epitope identified in four of the antigens, it was unclear whether only some or all of
558 these antigens were responsible for inducing the T cell responses. The HAD family of
559 proteins are phosphatases found in a wide range of prokaryotic and eukaryotic organisms,
560 identifiable by the presence of conserved sequence motifs (64). *Plasmodium falciparum* has
561 23 HAD-encoding genes, two of which have been implicated as targets for the experimental

562 therapeutic compound forsidomycin, which targets the methyl-erythrol phosphate pathway
563 of the apicomplast organelle (65). However, unlike the *T. parva* HAD proteins identified as
564 antigens, these *Plasmodium* proteins do not possess signal peptides and hence the former
565 are likely to have different biological functions, potentially involving interaction with host
566 proteins. The SVSP family, which has only been described in *Theileria*, consists of 85
567 members encoded by genes located in the telomeric regions of all four nuclear
568 chromosomes (66, 67), 14 of which were included in the peptide library. They encode a
569 short, conserved N-terminal region including a signal peptide, followed by a QP-rich central
570 region and a conserved C-terminus. Some contain a functional nuclear localisation signal
571 and some are expressed by only a proportion of cells in cultures of infected cells. However,
572 as with the HAD proteins, the functions of SVSPs remain unclear. Further studies are
573 required to explore whether additional members of the SVSP family, not included in the
574 peptide library, are recognised by parasite-specific T cells.

575 The results of the present study provide new insight into the antigenic basis of protective
576 immune responses against *T. parva* and have important implications for efforts to develop a
577 subunit vaccine. Early studies of *T. parva*-specific CD4 T cell clones revealed that some
578 clones were parasite strain-restricted (68), indicating polymorphism of the target antigens.
579 Moreover, some of the antigens identified in the current study, most notably Tp2 and Tp9,
580 are known to be highly polymorphic and result in strain-specificity of CD8 T cell responses
581 (12, 69, 70). However, many of the CD4 T cell antigens showed no or only limited
582 polymorphism. Hence, given the broad antigen specificity of CD4 T cells detected in
583 individual animals, it is unlikely that the CD4 T cell response contributes to the observed
584 parasite strain restriction of immunity to *T. parva*. Even in situations where polymorphic
585 antigens are responsible for a substantial component of the response, the panmictic
586 population structure of *T. parva* (71) arising from frequent sexual recombination during tick
587 passage (reviewed in 7), which allows independent segregation of alleles of different
588 antigens, favours cross-reactivity of the CD4 T cell responses to different parasite isolates.

589 With regard to vaccination, the findings have provided valuable information on which to base
590 selection of antigens suitable for vaccine development. As discussed earlier, there is
591 evidence that parasite-specific CD4 T cell responses are required for efficient activation of
592 memory CD8 T cells and *in vitro* studies suggest that such help requires antigens to be
593 displayed on the same antigen-presenting cells for recognition by CD4 and CD8 T cells (19).
594 This is clearly the case for *T. parva*-infected cells, which express both class I and class II
595 MHC proteins on their cell surface allowing activation of both CD4 and CD8 T cell responses
596 following infection. The evidence that both CD8 and CD4 antigens undergo intracellular
597 (endogenous) routes of antigen processing in infected cells implies that the same antigen
598 delivery systems can be used in a subunit vaccine to generate both CD4 and CD8 T cell
599 responses. In this regard, it is significant that immunisation of calves with recombinant adeno
600 and vaccinia viruses vectors expressing two of the identified antigens were found to induce
601 both CD4 and CD8 T cell responses reactive with parasitized cells and that the epitopes
602 recognised by the CD4 T cells were identical to those induced by infection with *T. parva*.
603 While induction of CD8 T cell responses by antigens expressed in viral vectors could
604 theoretically utilise help provided by CD4 T cells specific for the vector viruses, efficient
605 recall of such responses following parasite challenge will require vaccine-induced CD4 T
606 cells responses against epitopes presented by *T. parva*-infected cells. The present study has
607 shown that *T. parva*-infected cells display CD4 epitopes from a range of different parasite
608 proteins. Data on polymorphism will permit selection of antigens with conserved amino acid
609 sequences, to avoid strain specificity of CD4 T cell responses. Further studies are required
610 to determine the capacity of candidate antigens to induce CD4 T cell responses in animals of
611 different MHC genotypes when delivered in suitable vaccine vectors. Although responses of
612 immune animals to most of the antigens in the present study were only detected in certain
613 MHC genotypes, it is possible that immunisation with vaccine vectors will reveal
614 immunogenicity in a wider range of genotypes. Similar investigations are ongoing to identify
615 CD8 antigens with these properties. If different antigens prove optimal for induction of CD4

616 and CD8 T cell responses, it will be important to determine whether they can be delivered
617 separately or need to be incorporated into the same vaccine vector for optimal results.

618 In conclusion, this study has clearly demonstrated that CD4 T cell responses of cattle to *T.*
619 *parva* recognise multiple antigens presented on the surface of parasitized cells. Coupled
620 with information on sequence polymorphism of the antigens and evidence that viral vaccine
621 vectors expressing the antigens are capable of generating responses with the same fine
622 antigenic specificity as those induced in infected cattle, the results provide a valuable
623 resource for further studies to develop a subunit vaccine.

624

625 **Acknowledgements:** The authors wish to thank Professor Sarah Gilbert, The Edward Jenner
626 Institute, Oxford University for producing recombinant vaccinia and adeno viruses, and JPT
627 Peptide Technologies GmbH for advice on the peptide library. We are also grateful to Christina
628 Vrettou for expert technical assistance. We wish to dedicate this publication to Dr Niall
629 MacHugh, who initiated some of the early experiments reported in this study but unfortunately
630 passed away in 2017.

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870 Figure Legends:

871 Figure 1: Schematic illustration of parameters used to select proteins for production of a
872 synthetic peptide library for antigen screening, based on the predicted properties of *T. parva*
873 gene products: Secreted = proteins possessing a signal peptide; TM = transmembrane
874 domain; GPI = glycosylphosphatidylinositol anchor.

875 Figure 2: Results obtained from secondary screens of a subset of the *T. parva* peptide
876 library with CD4 T cells from immune cattle: A set of 78 peptide pools were used, including
877 all those detected as positive in primary screens of 514 pools, plus pools that were negative
878 in all screens. Cells from a *T. parva*-stimulated CD4 T cell line from each animal were tested
879 in duplicate wells. Reactivity of the CD4 T cells was assayed by testing the ability of
880 supernatants of peptide-stimulated cells to upregulate expression of surface class II MHC on
881 MDBK cells, as a measure of IFN- γ release. Results are shown for CD4 T cell lines from 3
882 MHC-homozygous animals – 1011 (DRB3 010:01), 4003 (DRB3 011:01) and 2824 (DRB3
883 016:01). Position 79 in each panel represents the result obtained with supernatants from
884 CD4 T cells incubated with MDBK cells alone. The contents of each peptide pool are shown
885 in supplementary Table S1.

886 Figure 3: Mapping of epitopes recognised by CD4 T cells: Results are shown for responses
887 to individual overlapping 18-mer peptides for five pools containing peptides for Tp18, Tp24,
888 Tp29, Tp30 and Tp32, using CD4 T cells from animals 2059, 3846, 2824, 2824 and 4003
889 respectively. The assays used cells from a *T. parva*-stimulated CD4 T cell line from each
890 animal, tested in duplicate wells. Responses were assayed as described in the legend for
891 figure 2. The final well in each panel (position 51) shows the response of T cells to MDBK
892 cells incubated with the respective peptide pool.

893 Figure 4: Class II MHC-restriction of CD4 T cells specific for *T. parva* epitopes: Presentation
894 of individual epitopes by MHC class II DR was examined by measuring responses to
895 Chinese hamster ovary (CHO) cells transfected with the DRB3 010:01 or DRB3 011:01
896 alleles, which had been pre-incubated with peptide and washed prior to incubation with CD4
897 T cells. CD4 T cell lines from animals homozygous for DRB3 010:01 or DRB3 011:01, were
898 assayed in two experiments for each (i and ii). The assays used cells from a *T. parva*-
899 stimulated CD4 T cell line from each animal, tested in duplicate wells. Reactivity of the CD4
900 T cells was assayed by testing the ability of supernatants of stimulated T cells to upregulate
901 expression of surface class II MHC on MDBK cells, as a measure of IFN- γ release. Results
902 are shown for responses to peptide added directly to CD4 T-cells and auto-presented
903 (positive control), responses against peptide pre-incubated with CHO cells expressing an
904 irrelevant BoLA-DR molecule (negative control) and responses against peptides pre-
905 incubated with CHO cells expressing the relevant BoLA-DR molecule. * $p < 0.05$ compared to
906 responses to cells expressing the irrelevant DR.

907 Figure 5: Class II MHC DR- or DQ-restriction of CD4 T cells specific for *T. parva* epitopes:
908 Inhibition of epitope recognition by monoclonal antibodies specific for bovine DR (IL-A21)
909 and DQ (CC158) was examined by measuring IFN- γ release in supernatants of peptide-
910 stimulated CD4 T cells using a specific ELISA. Cells from a *T. parva*-stimulated CD4 T cell
911 line from each animal were tested in duplicate wells. Results are shown for two DR-restricted
912 (Tp16₁₀₃₋₁₂₀ and Tp19₈₃₅₋₈₅₂) and two DQ-restricted epitopes (Tp2₇₆₋₉₂ and Tp15₅₁₇₋₅₃₄).
913 Responses to peptide in the absence of added antibody are shown in grey. For each
914 epitope, inhibition by the respective antibody was observed compared to the non-inhibitory
915 antibody $p < 0.01$.

916 Figure 6: Amino acid sequence variation across coding sequence and location of
917 epitopes: Average pairwise difference per non-synonymous site between sequences (π_{NS}) is
918 shown for six representative antigens. π_{NS} was calculated using a sliding window of 30 sites,

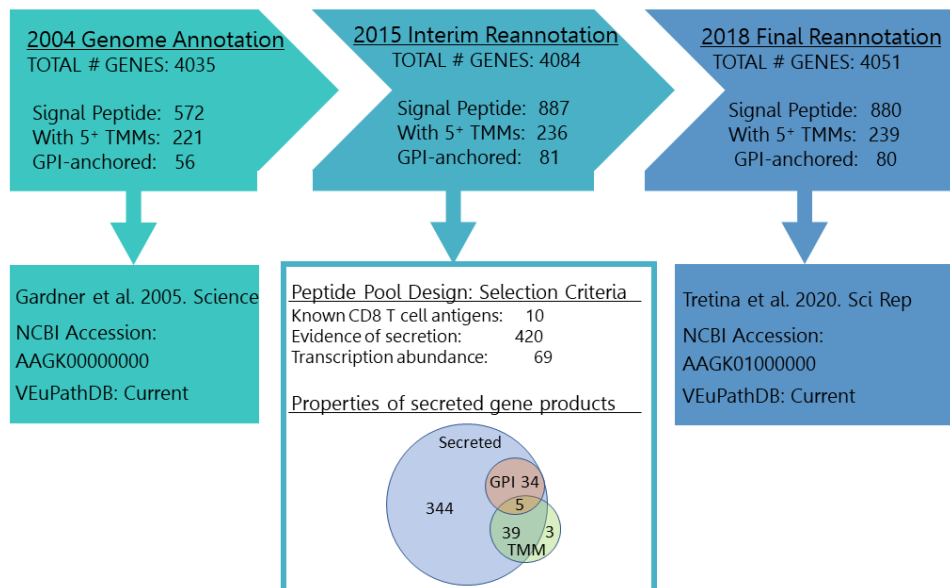
919 with a five-site step. The π_{NS} plot is interrupted in regions of the alignment with indels
 920 between alleles. Repeat regions are shown in grey below the plot. The location of epitopes
 921 shown in Table 3 is marked by MHC genotype in yellow (DR 10:01), green (DR 11:01) and
 922 blue (DR 16:01).

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926 Figure 1



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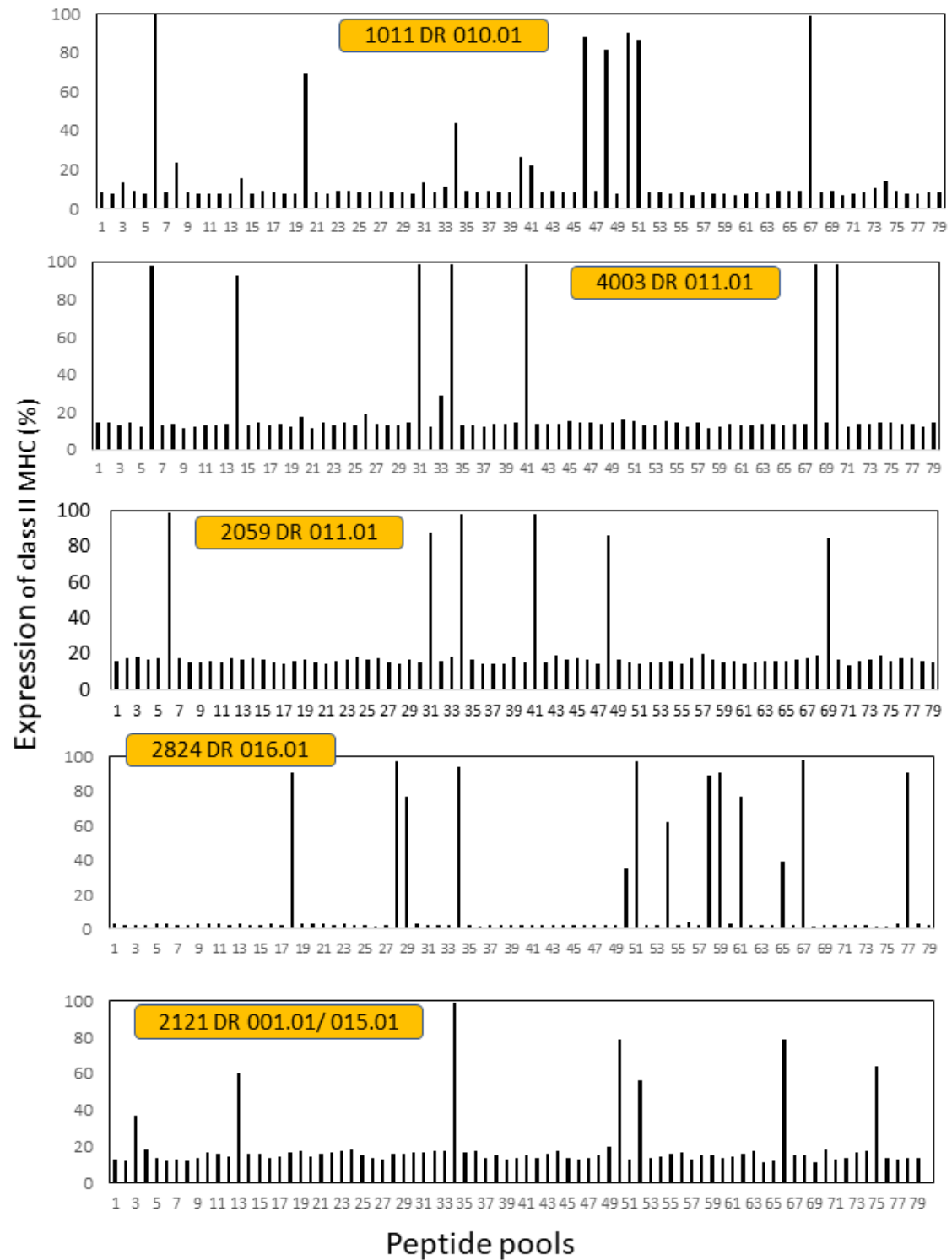
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Figure 2



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942 Figure 3

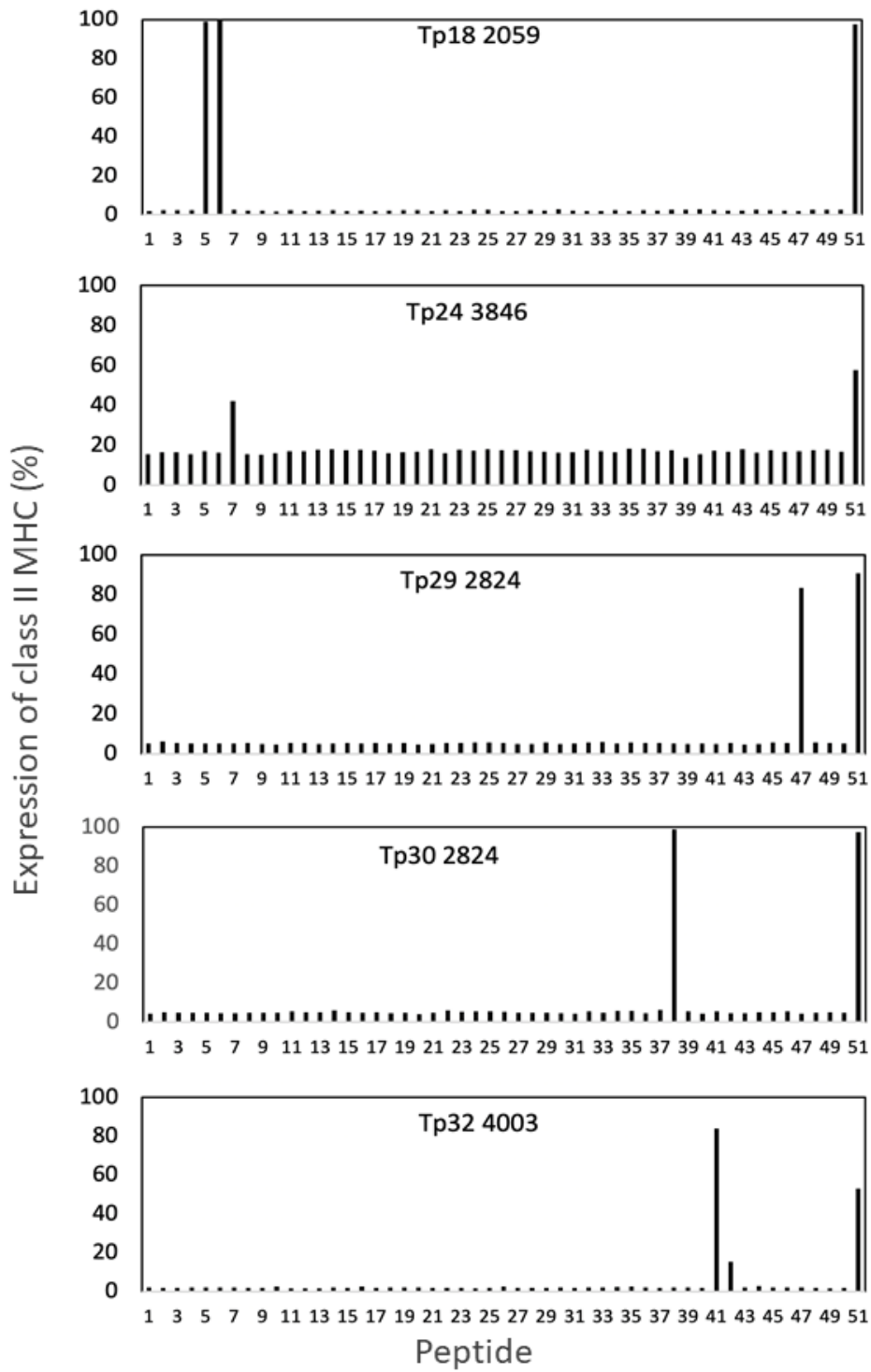
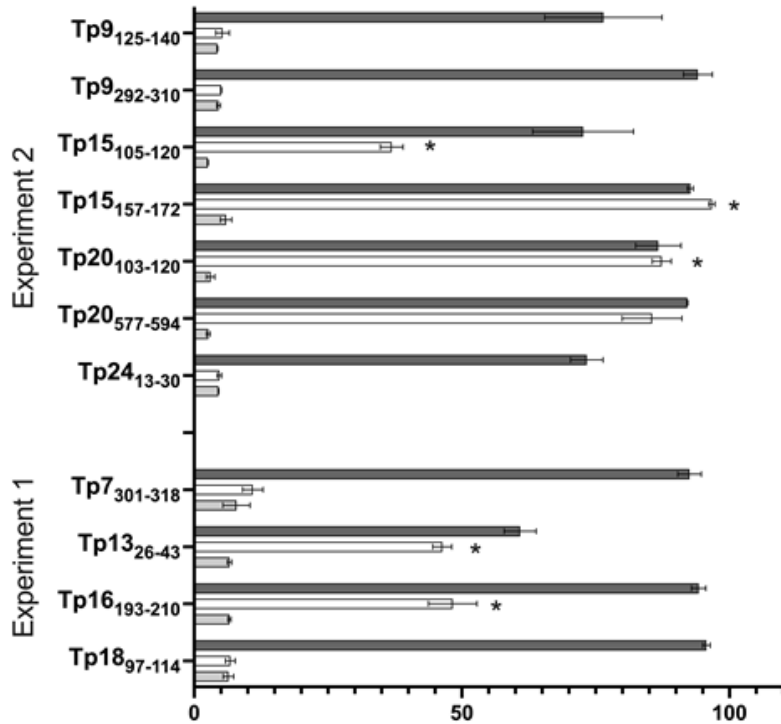


Figure 3

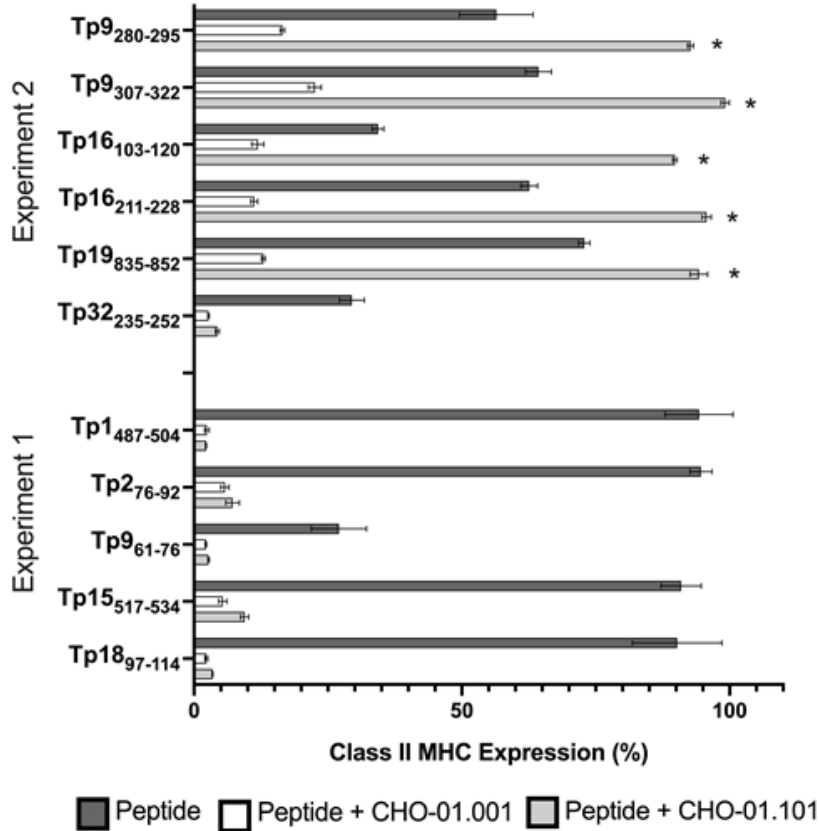
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(a) DR 01.001 Figure 4



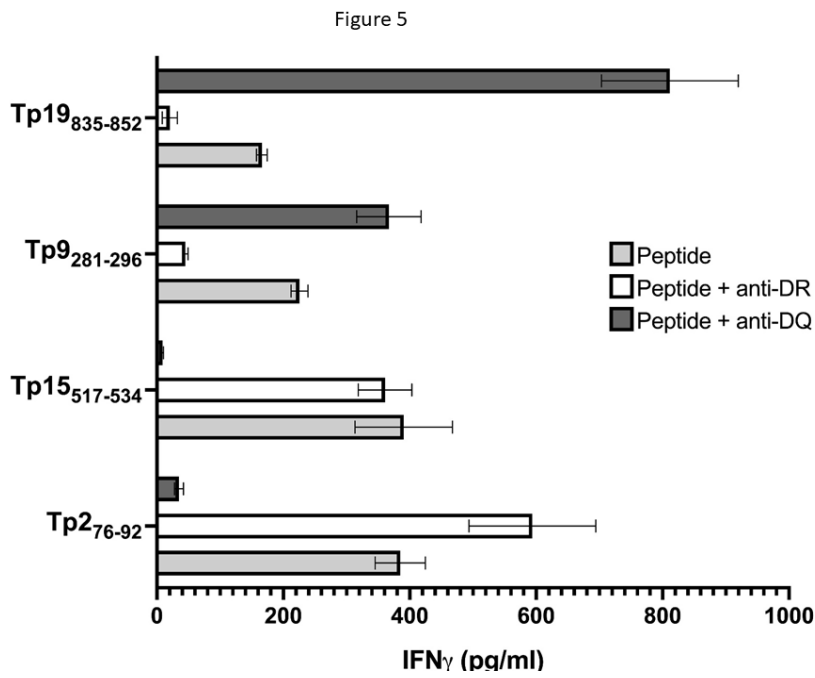
(b) DR 01.101



Class II MHC Expression (%)

Peptide
 Peptide + CHO-01.001
 Peptide + CHO-01.101

947 Figure 5

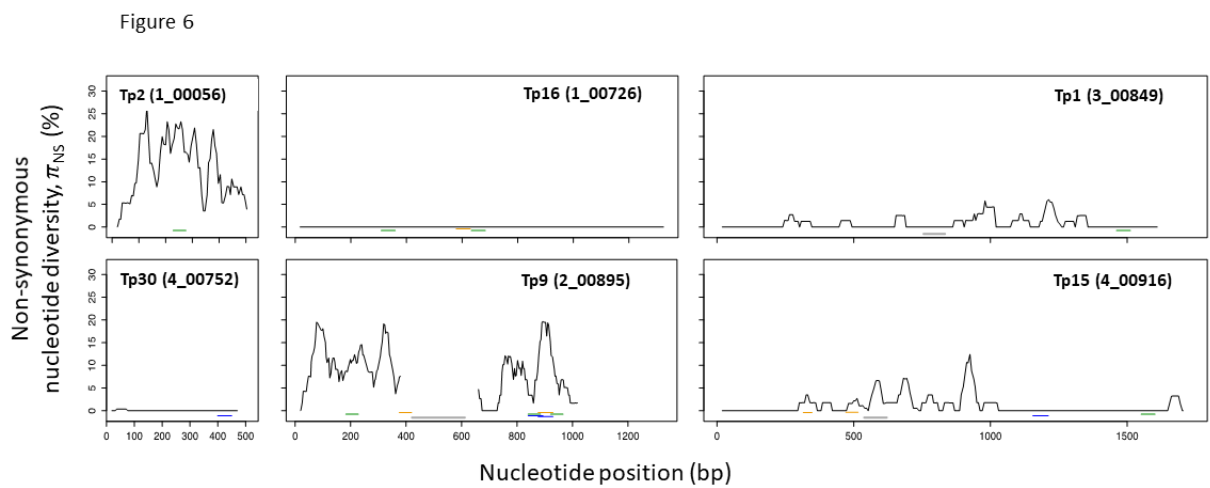


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951 Figure 6



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953 Table 1: Summary of antigens recognised by CD4 T cells from 12 cattle immunised against *Theileria parva*

Antigen ¹	Gene ID ²	CD8 ³ antigen	Animals and MHC types (Class I serotypes, class II DR genotypes)											
			598 A15/A17 11:01/16:01	2121 A10/A12 01:01/15:01	2786 A12/A20 12:01/15:01	2759 A13/A19 07:01/27:01	2756 A13/N5 14:01/27:03	2824 A19/A19 16:01/16:01	1011 A10/A10 10:01/10:01	3846 A10/A10 10:01/10:01	2059 A11/A15 11:01/11:01	4003 A14/A14 11:01/11:01	605 A14/A14 09:01/09:01	641 A18/A18 20:02/20:02
Tp2	1_0056	yes	+			+	+				+	+		
Tp16	1_0726		+						+	+	+	+		+
Tp36 (gp34)	1_0939									+				
Tp14	1_1074		+					+						+
Tp17	1_1077		+					+						
Tp21	1_1078		+					+						
Tp22	1_1081		+					+						
Tp23	1_1082		+					+						
Tp24	1_1182			+						+				+
Tp25	1_1225						+							+
Tp26	2_0010						+							
Tp32	2_0123	yes								+	+			
Tp27	2_0243						+							
Tp7	2_0244	yes							+	+				
Tp9	2_0895	yes	+	+	+		+	+	+	+	+	+		
Tp28	2_0958				+					+				
Tp33	3_0263	yes		+										+
Tp13	3_0655						+		+					
Tp1	3_0849	yes		+			+		+		+			
Tp20 (p150)	3_0861			+	+			+	+	+			+	+
Tp18 (PIM)	4_0051								+	+				
Tp19 (p104)	4_0437								+	+	+	+	+	+
Tp29	4_0683						+	+						
Tp30	4_0752							+						
Tp15	4_0916							+	+	+		+		+

Tp31	4_0917			+	+			+						
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954 ¹ Previously used terminology shown in parenthesis.

955 ² Gene identifier is presented in format X_YYYY, where X is the nuclear chromosome number and YYYY represents the locus number.

956 ³ CD8 antigens Tp1, Tp2 and Tp7 reported by Graham et al., 2006 and Tp9 by Hemmink et al., 2016; Tp32 and Tp33 detected in recent antigen screens (N. D.
957 MacHugh, unpublished data.

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975 Table 2: Properties of antigens recognised by CD4 T cells from cattle immunised against *Theileria parva*

Ag ^a	Gene ID ^b	Protein					Gene Product ^f	Host Homologues		N ^h	π_{NS} (%) ⁱ	π_S (%) ^j	Expression rank ^k
		Length (aa)	Size (kD)	Sig P ^c	TM ^d	GPI ^e		Bovine	Simil. (%) ^g				
Tp2	1_0056	174	19.1	+				-	-	16	14.5	21.8	229
Tp16	1_0726	448	49.5				Elongation factor Tu GTP binding domain	DAA23235.1	72.02	15	0	1.14	4
Tp36	1_0939	307	36.4	+		+	Schizont surface protein	-	-	17	0.24	1.94	984
Tp14	1_1074	318	36.0	+			haloacid dehalogenase-like hydrolase	-	-	17	0.00	0.00	90
Tp17	1_1077	292	32.9	+			haloacid dehalogenase-like hydrolase	-	-	13	0.07	0.25	3062
Tp21	1_1078	304	34.2	+			haloacid dehalogenase-like hydrolase	-	-	16	0.02	0.00	2129
Tp22	1_1081	307	34.4	+			haloacid dehalogenase-like hydrolase	-	-	13	1.88	3.46	3331
Tp23	1_1082	303	34.5	+			haloacid dehalogenase-like hydrolase	-	-	13	0.04	0.24	3476
Tp24	1_1182	321	34.9				lactate/malate dehydrogenase	DAA13962.1	31.76	17	0.02	1.44	14
Tp25	1_1225	433	48.8	+			SVSP family protein	-	-	12	0.18	0.39	512
Tp26	2_0010	450	52.0	+			SVSP family protein	-	-	15	0.44	0.14	1851
Tp32	2_0123	400	45.5				DEAD/DEAH box helicase	DAA18212.1	65.33	17	0.00	0.00	34
Tp27	2_0243	818	93.1				Heat shock protein homolog pss1	DAA27494.1	32.48	14	0.09	1.99	520
Tp7	2_0244	721	83.7				HSP90	DAA17282.1	66.11	12	0.03	0.72	5
Tp9	2_0895	334	34.7	+				-	-	13	9.08	14.24	8
Tp28	2_0958	675	76.4	+			SVSP family protein	-	-	14	0.95	2.25	2475
Tp33	3_0263	788	90.7	+			Schizont-associated, in complex with CLASP	-	-	15	0.07	0.00	1046
Tp13	3_0655	157	17.2					-	-	17	0.04	2.00	82
Tp1	3_0849	543	61.4	+				-	-	17	0.78	0.65	622
Tp20	3_0861	1452	164.9	+			P150 - sporozoite microspheres, schizont secreted	-	-	13	0.52	1.21	287
Tp18	4_0051	480	52.4	+		+	PIM - sporozoite microspheres, schizont surface	-	-	9	2.53	1.35	3
Tp19	4_0437	924	103.6	+		+	p104 – sporozoite rhoptry, schizont surface	-	-	14	0.11	0.07	61

Tp29	4_0683	655	72.7	+		78 kDa glucose-regulated protein	DAA24281.1	65.62	14	0.04	0.83	160
Tp30	4_0752	162	18.3			Ribosomal S27a family protein	DAA24675.1	55.56	17	0.03	0.90	28
Tp15	4_0916	574	64.8	+		SVSP family protein	-	-	17	1.07	2.04	844
Tp31	4_0917	528	60.2	+		SVSP family protein	-	-	15	0.33	0.58	2298

976 ^a Ag: Antigen; ^b Gene: Proxy for gene identifier in the genome of the *T. parva* reference strain Muguga, where the first digit stands for chromosome number
977 and the last four digits to the locus number in the original and the updated genome annotations (respectively, Gardner et al. 2005 and Tretina et al. 2020); ^c
978 Sig P: Presence of a signal peptide targeting the protein to the secretory pathway with high reliability, as determined with TargetP v1.1; ^d TM: Presence of
979 one or more trans-membrane domains; ^e GPI: Presence of a predicted GPI anchor, as determined by GPI-SOM and/or predGPI; ^f Properties relating to
980 parasite highlighted in red; ^g Simil: Percent amino acid similarity over segment of protein aligned to host protein; ^h Number of alleles used to estimate
981 synonymous and non-synonymous nucleotide diversity; ⁱ Non-synonymous nucleotide diversity: average number of non-synonymous mutations per non-
982 synonymous site among *T. parva* strains infecting cattle, per 100 sites; ^j Synonymous nucleotide diversity: average number of synonymous mutations per
983 synonymous site among cattle-infecting *T. parva*, per 100 sites; ^k Expression Rank: Expression of all *T. parva* genes in the schizont stage (Tretina et al 2016)
984 was ranked, with 1 being the most expressed and 4051 the least expressed, and genes assigned the corresponding rank.

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999 Table 3: CD4 T cell epitopes¹ identified in *Theileria parva* antigens recognised by cattle homozygous for the DR 1001, 1101 and 1601 alleles

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Antigen	Gene ID	DR 01.001 ⁺ animals epitope	DR 01.101 ⁺ animals epitope	DR 01.601 ⁺ animal epitope	MHC restriction ²	
					DR	DQ
Tp2	1_0056		76-LETLFGKHGLGGISKDC ₋₉₂			+
Tp16	1_0726	193-GFLGDNMIDKSDKMPWYK ₂₁₀	103-ITGTSQADVAMLVVPAES ₋₁₂₀ 211-GKILVEALDLMEPKRPV ₋₂₂₈		+	
					+	
					+	
Tp14	1_1074			55-KYFAIDIDGTFFIKD ₋₆₇ 76-NIAAFKRLQDAGVLPFF ₋₉₂	+	
					+	
Tp17	1_1077			30-KYFAIDIDGTFHIKD ₋₄₄	+	
Tp21	1_1078			24-IYFGVDIDGTFYVED ₋₃₈	nt	
Tp22	1_1081			34-KYFAIDIDGTFHIKD ₋₄₄	nt	
Tp23	1_1082			40-KFFAIDIDGTFYIND ₋₅₄	nt	
Tp24	1_1182	13-GSGNIGGIMGYLSQLTEL ₋₃₀				+
Tp32	2_0123		235-ELTLEGIKQFYILIDKEY ₋₂₅₂		+	
Tp7	2_0244	301-NEEYAAFYKNLTNDWEDH ₃₁₈			+	
Tp9	2_0895	125-GPYGQAGYVGPQGA VG ₋₁₄₀ 292-GDYAVKVLVFPIGFKEKTI ₋₃₁₀	61-TKQDLDAKFPGMKSK ₋₇₆ 280-YDGEKVWSLEVGGDYA ₋₂₉₅ 307-EKTIEITFIGGEKEIY ₋₃₂₂	281-DGEKVWSLEVGGDYAV ₋₂₉₆ 292-GDYAVKVLVFPIGFKEKTI ₋₃₁₀		+
						+
					+	+
					+	
Tp13	3_0655	26-GRVSNYVTYAKLLSNGI ₋₄₃			+	
Tp1	3_0849		487-SIVNVYGKNDEPLSYAPS ₋₅₀₄			+
Tp20	3_0861	103-QEILYYKWEKHGFVKETY ₋₁₂₀ 577-PLSGYHVRYVNYGKVIMW ₋₅₉₄		103-QEILYYKWEKHGFVKETY ₋₁₂₀ 451-FNKFDMLHDGVYSSPVP ₋₄₆₈ 1351-CKANNPVVYIKAGDKTVW ₋₁₃₆₈	+	
					+	
					+	
					+	
Tp18	4_0051	97-QQGPDPQPQIQEPSGPVQ ₁₁₄	97-QQGPDPQPQIQEPSGPVQ ₁₁₄			+

Tp19	4_0437		835-KSFDDLTTVELAPEPKAS-852		+	
Tp29	4_0683			499-QIEVTFNIDTNGILSVTA-516	+	
Tp30	4_0752			133-NCGRGVFMAAHNNRITYCG-150	+	
Tp15	4_0916	105-GTYQHYGPPVFPPQPE-116 157-GIQYVPYQTLQIPQPQ-172	517-RNQVWIKTASEGFPSSM-534	385-IEMTEKEYKIIVDSRF-400	+	
					+	
					+	
						+

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1002 ¹The location of the epitopes was determined by screening overlapping 18-mer peptides (overlapping by 12 residues) for the respective positive antigen,
1003 using an interferon- γ release assay as described by Hart et al., 2017. In some cases, further truncated peptides were tested.

1004 ² The class II restriction of T cells specific for the epitopes was determined using two assays, first by testing recognition of the peptides pre-incubated with
1005 CHO cells expressing the 1001 or 1101 class II DR alleles and, second by testing the ability of monoclonal antibodies specific for bovine DR or DQ (IL-A21 and
1006 CC158 respectively) to inhibit recognition of the peptides. Nt = not tested.

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1019 Table 4: CD4 and CD8 T cell responses of 4 animals immunised with recombinant adenovirus and vaccinia viruses expressing the
 1020 Tp9, Tp10 and Tp15 *T. parva* antigens.

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Antigens ¹	T cell lines and MHC genotypes ²							
	2750 MHCI - A10/A14, DR - 01.001/01.101		2733 MHCI – A11/A14 DR - 01.101/01.401		2751 MHCI – A14/A15 DR - 01.101/01.401		2758 MHCI – A10/A11 DR - 01.001/01.101	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Control	3 (0.3)	18 (1.1)	6 (1.5)	15 (0.2)	3 (1.3)	nt	6 (1.6)	3.5 (0.4)
Tp9	98 (1.5)**	95 (2.0)**	99 (0.7)**	73 (3-5)**	99 (0.2)**	nt	99 (0.2)**	97 (0.9)**
Tp10	97 (1.9)**	98 (2.8)**	33 (2.9)*	10 (0.9)	90 (4.0)**	nt	15 (2.7)*	32.6 (1.5)**
Tp15	99 (0.3)**	16 (2.0)	88 (5.4)**	14 (0.6)	29 (3.0)**	nt	85 (3.1)**	3.8 (0.7)

1022 ¹ The antigens consisted of pools of overlapping 18-mer synthetic peptides for each antigen. Controls represent responses to a peptide pool not
 1023 recognised on these MHC backgrounds

1024 ² Responses were measured by testing the ability of supernatants from antigen-stimulated T cells to up-regulate expression of surface class II
 1025 MHC on MDBK cells. Results are expressed as the % of class II MHC-positive cells, showing the mean (and standard deviation) for duplicates
 1026 of each assay. * p<0.05, ** p<0.01.

1027 nt – Not tested.

1028 Table 5: Response to known Tp9 and Tp15 epitopes of CD4 T cells from an animal (2750) vaccinated with the Tp9 and Tp15
 1029 antigens expressed in recombinant adenovirus and vaccinia vaccine vectors

Antigen	Responses of CD4 T cell lines (MHC DR type) ¹		
	3846 (01.001/01.001)	4003 (01.101/01.101)	2750 (01.001/01.101)
Control ²	9 (1.6)	5 (0.6)	6 (0.6)
Tp9 ₆₁₋₇₆		39 (3.5)*	5 (0.4)
Tp9 ₁₂₅₋₁₄₀	98 (1.3)**		94 (2.1)**
Tp9 ₂₈₀₋₂₉₅		99 (0.1)**	97 (1.1)**
Tp9 ₂₉₂₋₃₁₀	63 (8.8)*		82 (3.9)**
Tp9 ₃₀₇₋₃₂₂		99 (1.0)**	99 (0.4)**
Tp15 ₁₀₅₋₁₂₀	96 (2.7)**		5 (0.5)
Tp15 ₁₅₇₋₁₇₂	99 (0.2)**		36 (4.1)*
Tp15 ₅₁₇₋₅₃₄		90 (3.9)**	33 (5.8)*

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1031 ¹ The 3846 and 4003 CD4 T cell lines were from *T. parva*-immune MHC-homozygous animals, which were used for antigen identification.
 1032 Responses were measured by testing the ability supernatants from peptide-stimulated T cells to up-regulate expression of surface class II MHC
 1033 on MDBK cells. Results are expressed as the % of class II MHC-positive cells, showing the mean (and standard deviation) for duplicates of
 1034 each assay. * p<0.05, ** p<0.01.

1035 ² Controls represent responses to a peptide not recognised on either of these MHC backgrounds