The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2021

CD4 T Cell Responses to *Theileria parva* in Immune Cattle Recognize a Diverse Set of Parasite Antigens Presented on the Surface of Infected Lymphoblasts

W. Ivan Morrison; ... et. al

J Immunol (2021) 207 (8): 1965–1977.
https://doi.org/10.4049/jimmunol.2100331

CD4 T Cell Responses to *Theileria parva* in Immune Cattle Recognize a Diverse Set of Parasite Antigens Presented on the Surface of Infected Lymphoblasts

W. Ivan Morrison,* Adriana Aguado,* Tara A. Sheldrake,* Nicholas C. Palmateer,† Olukemi O. Ifeonu,† Kyle Tretina,† Keith Parsons,‡ Emilio Fenoy,§ Timothy Connelley,* Morten Nielsen,§,¶ and Joana C. Silva†,‖

Parasite-specific CD8 T cell responses play a key role in mediating immunity against *Theileria parva* in cattle (*Bos taurus*), and there is evidence that efficient induction of these responses requires CD4 T cell responses. However, information on the antigenic specificity of the CD4 T cell response is lacking. The current study used a high-throughput system for Ag identification using CD4 T cells from immune animals to screen a library of ~40,000 synthetic peptides representing 499 *T. parva* gene products. Use of CD4 T cells from 12 immune cattle, representing 12 MHC class II types, identified 26 Ags. Unlike CD8 T cell responses, which are focused on a few dominant Ags, multiple Ags were recognized by CD4 T cell responses of individual animals. The Ags had diverse properties, but included proteins encoded by two multimember gene families: five haloacid dehalogenases and five subtelomere-encoded variable secreted proteins. Most Ags had predicted signal peptides and/or were encoded by abundantly transcribed genes, but neither parameter on their own was reliable for predicting antigenicity. Mapping of the epitopes confirmed presentation by DR or DQ class II alleles and comparison of available *T. parva* genome sequences demonstrated that they included both conserved and polymorphic epitopes. Immunization of animals with vaccine vectors expressing two of the Ags demonstrated induction of CD4 T cell responses capable of recognizing parasitized cells. The results of this study provide detailed insight into the CD4 T cell responses induced by *T. parva* and identify Ags suitable for use in vaccine development. ***The Journal of Immunology*****, 2021, 207: 1965–1977.**

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

Strategies adopted for development of subunit vaccines have built on knowledge of immune responses in animals immunized with live parasites. A large body of evidence indicates that immunity against *T. parva* is mediated by T cell responses against the intralymphocytic schizont stage of the parasite and that parasite-specific CD8 T cells play a central role in immunity (reviewed in Ref. 7). Notably, it has been possible to confer immunity against parasite challenge

*The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian, United Kingdom; †Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD; ‡Institute for Animal Health, Berkshire, United Kingdom; §Biotechnological Research Institute, National University of San Martin, Buenos Aires, Argentina; ¶Department of Health Technology, Technical University of Denmark, Kongens Lyngby, Denmark; and ‖Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD

ORCIDs: 0000-0003-1875-0742 (T.A.S.); 0000-0002-4307-8049 (N.C.P.); 0000-0002-1740-2730 (K.T.); 0000-0001-9121-7149 (E.F.); 0000-0001-7885-4311 (M.N.); 0000-0001-6502-7026 (J.C.S.).

Received for publication April 6, 2021. Accepted for publication July 22, 2021.

This work was supported by a grant awarded by the Bill and Melinda Gates Foundation jointly with the United Kingdom Department for International Development (OPP1078791) and a Biotechnology and Biological Sciences Research Council Institute Strategic Programmes Grant (BB/J004227/1).

Address correspondence and reprint requests to Prof. W. Ivan Morrison, The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, 3 Winterfield Place Belhaven, Dunbar, East Lothian EH42 1QQ, U.K. E-mail address: ivan.morrison@roslin.ed.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: CHO, Chinese hamster ovary; HAD, haloacid dehalogenase; MDBK, Madin-Darby bovine kidney; MVA, modified vaccinia Ankara; π_{NS}, average number of nonsynonymous differences per nonsynonymous site; π_S, average number of synonymous differences per synonymous site; SVSP, subtelomere-encoded variable secreted protein.

by adoptive transfer of purified actively responding CD8 T cells from immune to naive identical twin calves (8). Moreover, CD8 T cell responses in cattle immunized with a single parasite isolate are frequently strain-restricted, which has been shown to correlate with susceptibility to subsequent challenge with heterologous parasite strains (9). A striking feature of the parasite-specific CD8 T cell response is that the response in individual animals is focused on a few highly dominant Ags, which are often polymorphic (10). This immunodominance is critical in determining the strain restriction of immunity.

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

The adoptive transfer studies referred to above used CD8 T cells collected during the active response of immune calves to challenge with *T. parva*. Hence, it remains unclear whether other components of the immune response are required for efficient activation of memory CD8 T cells following parasite challenge. Help from CD4 T cells is required for efficient induction of CD8 T cell responses against many viral infections (reviewed in Ref. 14). Moreover, studies of responses to *Plasmodium* parasites using murine models have shown that the induction and maintenance of CD8 T cell responses to the pre-erythrocytic stage of the parasite are strongly dependent on the presence of specific CD4 T cells (15–17). *T. parva*–infected lymphoblasts express MHC class II on their surface, and immune animals exhibit strong parasite-specific CD4 T cell responses against parasitized cells (7, 18). However, their role in immunity has received relatively little attention. Bovine CD8 T cells do not appear to produce IL-2 upon activation and in vitro studies have indicated that their ability to respond following Ag recognition is dependent on production of soluble growth factors by other cell types. Experiments using different combinations CD4 and CD8 T cells purified from genetically identical *T. parva*–immune or –naive twin calves have demonstrated that optimal proliferation of the immune CD8 T cells required the presence of Ag-specific CD4 T cells (19). Moreover, the results indicated that both cell types had to recognize Ag presented by the same *T. parva*–infected cells. These observations suggest that successful vaccination against *T. parva* with defined Ags requires induction of both CD4 and CD8 T cell responses of appropriate specificities and functional activities. Although a series of early studies identified crude antigenic fractions of parasitized cells recognized by *T. parva*–specific CD4 T cells (18, 20–22), the precise antigenic specificities of the responding T cells have not been defined.

Because the development of efficacious vaccines against *T. parva* is likely to require inclusion of CD4 as well as CD8 T cell Ags to provide robust protection, the current study set out to identify the parasite Ags recognized by CD4 T cell responses in cattle immunized against *T. parva* by infection and treatment. Ags were identified by undertaking a high-throughput screen with CD4 T cells from immune cattle of diverse MHC types, using a library of peptides representing 499 parasite proteins (~12.5% of the parasite proteome). The results demonstrate that, in contrast to the narrow specificity of CD8 T cell responses, CD4 T cells from immune cattle recognize multiple Ags presented by parasite-infected cells and that such responses can readily be induced using viral vaccine vectors.

Materials and Methods

Animals and immunization

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

Animals used for Ag screening were immunized with the Muguga isolate of *T. parva* by infection with cryopreserved sporozoites and simultaneous treatment with a slow-release formulation of oxytetracycline, as described previously (2). Additional animals were immunized with selected Ags expressed individually in recombinant human adenovirus and vaccinia virus (modified vaccinia Ankara [MVA] strain) vectors. Animals received 2×10^9 IU of each adenovirus followed 8 wk later by 5×10^8 PFU of each vaccinia virus, both administered i.m.

T cell lines

All cultures of parasitized cells and T cells were conducted in RPMI 1640 medium supplemented with 10% FBS, 2-mM L-glutamine, 5×10^{-5} M 2-ME, and penicillin and streptomycin. Cell lines infected with the Muguga stock of *T. parva*, from which the reference genome sequence was derived (26), were generated by infection of PBMCs in vitro with sporozoites (27). A parasite-specific CD4 T cell line was generated from each immune animal as described previously (28); briefly, PBMCs were stimulated with irradiated autologous *T. parva*–infected cells three times at weekly intervals, followed by depletion of CD8 and γδ T cells by Ab and complement–mediated lysis. In some cases, the cultures were subjected to a further positive selection step involving cell sorting of cells stained with a CD4-specific mAb (IL-A11) using an FACSAria cell sorter (BD Biosciences, Oxford, U.K.). Purified CD4 T cells (>98%) were maintained by stimulation at 7–10-d intervals with equal numbers of irradiated *T. parva*–infected cells in medium containing 50 U/ml recombinant human IL-2 (Chiron, Emeryville, CA).

Ag screening

A library of synthetic peptides representing 499 selected *T. parva* gene products was used for Ag screening. The gene products included in the library are listed in Supplemental Table I. This library consisted of 40,921 18-mer peptides overlapping by 12 residues, produced as a series of 819 pools each containing 50 contiguous peptides. Peptides were supplied by JPT Peptide Technologies GmbH (Berlin, Germany) and produced by their Microscale system, which results in an additional glycine residue at the C terminus of each peptide. Ag screening was conducted in 96-well round-bottom plates by incubation of each peptide pool with 2×10^4 CD4 T cells/well in a total volume of 150 μl medium, each peptide at a final concentration of 2 μg/ml. CD4 T cells harvested 9 to 10 d after antigenic stimulation were used for Ag screening to ensure low background levels of cytokine secretion. Because of the large numbers of CD4 T cells required for each screen, they were conducted in single wells. After incubation with peptide at 37°C for 48 h, culture supernatants collected from the wells were assayed for IFN-γ using a biological assay performed as described previously (29), based on the ability of the IFN-γ in culture supernatants to upregulate surface expression of MHC class II on Madin-Darby bovine kidney (MDBK) cells. The MDBK cells were harvested after 48 h and MHC class II expression assessed by flow cytometry following immunofluorescence staining with a class II DR-specific mAb (IL-A21). Results are expressed as the percentage of MDBK cells expressing surface class II.

For each T cell line, the screening assay was repeated with a limited set of peptide pools (Supplemental Table II) comprising all pools that were positive in the initial screen, other pools that contained peptides from Ags represented in the positive pools, and an additional 15 randomly selected pools that gave negative results with all T cell lines. This secondary screen, as well as all subsequent assays of the positive Ags, were conducted in duplicate wells. Only supernatants that resulted in at least 20 percentage points above the background levels of class II expression in both the primary and secondary screens were considered positive (all were statistically significant at $p < 0.01$ in the secondary screen). The 50 peptides in each pool sometimes originated from a single gene product, but in other cases were derived from two or occasionally three gene products. In the latter cases, subcomponents of the pools were rescreened in duplicate to determine which gene product gave rise to the positive response.

Epitope localization and MHC restriction

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

Two assays were used to determine whether peptides were presented by class II DR or DQ MHC proteins: a first assay used Chinese hamster ovary (CHO) cells expressing either of two defined bovine DR alleles, namely DR

010:01 and DR 011:01, known to be expressed by MHC-homozygous animals used in the study. These cells were produced by transfecting CHO cells with a construct containing cDNAs encoding both the DR α- and β-chains linked by an oligonucleotide from foot and mouth disease virus, which encodes a self-cleaving peptide (30). Cells transfected with this construct were stained with mAb IL-A21 and positive cells purified by cell sorting were expanded, and, after checking for DR expression, aliquots of the cells were cryopreserved prior to use. For the T cell assay, resuscitated CHO cells were distributed in 96-well U-bottom plates at 10^4/well, and duplicate wells were incubated with a range of concentrations of peptide (2 μg to 100 ng/ml) or medium for 2 h. The cells were then washed twice with culture medium and T cells added to the wells at 10^4/well. After incubation at 37°C for 48 h, supernatants were harvested and assayed for IFN-γ using the biological assay.

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

Properties of gene products

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

Polymorphism

For each locus, the orthologous allele was obtained from the draft genome assembly of 17 *T. parva* strains isolated from cattle from across the range of the distribution of the parasite. The genome sequence of each strain was generated as described before (39). When alleles were mostly or completely missing from the draft assembly, the strain was removed from the respective data set. When only a few nucleotides were missing in the 3′ or 5′ end, the sequence was kept in the data set, and the missing nucleotides were assumed to be identical to the haplotype that was otherwise identical to it. The nucleotide sequences were aligned using Muscle (40) and manually curated in Mesquite (41). Nucleotide diversity per site averaged across each gene was estimated for silent sites (synonymous; average number of synonymous differences per synonymous site [π_S]) and amino acid–changing sites (nonsynonymous; average number of nonsynonymous differences per nonsynonymous site [π_{NS}]) using DnaSP (42). In addition, π_{NS} was estimated across sliding windows of nonsynonymous sites, with width and step, respectively, of 30 and 5 nonsynonymous sites. Regions of low complexity and micro- and mini-satellites were identified using Tandem Repeats Finder (43). Plots of nucleotide diversity were drawn using R (44).

Statistical analysis

Data are presented as means and SDs. Statistical analyses were performed using Student *t* test. Statistically significant differences compared with the control are indicated by asterisks: $*p < 0.05$, $**p < 0.01$.

Results

Selection of parasite proteins for generating the peptide library

The approach used for selection of the parasite gene products to produce the peptide library for Ag screening is illustrated in Fig. 1, and the content of the library is listed in Supplemental Table I. Selection of proteins was based predominantly on the possession of a predicted signal peptide, indicating likely secretion from the intracellular parasite (45), and/or high abundance of transcription of the genes in infected lymphoblasts. Ten proteins previously shown to be CD8 T cell Ags were included (11, 12). A reannotated version of the *T. parva* genome was used to produce the parasite proteome sequence (46), from which 420 proteins highly likely targeted for the secretory pathway were selected; 39 of these have a likely GPI anchor and 47 have 5 or more transmembrane helices, with some overlap among those sets (Fig 1). An additional 69 proteins were selected based on abundance of transcription.

Screening of the peptide library

A *T. parva*–specific CD4 T cell line from each of the 12 immunized cattle was used for Ag screening. The donor cattle represented 12 different class II DRB3 alleles and included animals that were homozygous for 5 different MHC haplotypes (Table I). Pairs of cattle homozygous for DR 010.01 and DR 011.01 had been produced by father-daughter mating and, although they were not typed for class II DQ, are also highly likely to share the same DQ alleles. Screening of the peptide library (819 peptide pools representing 499 parasite proteins) with these CD4 T cell lines resulted in the identification of positive peptide pools in all animals; 32 of the pools yielded positive results. A secondary screen of all positive pools was used to confirm positive results and further screens of the subcomponents of pools that contained peptides from more than one parasite protein were carried out. The results identified a total of 26

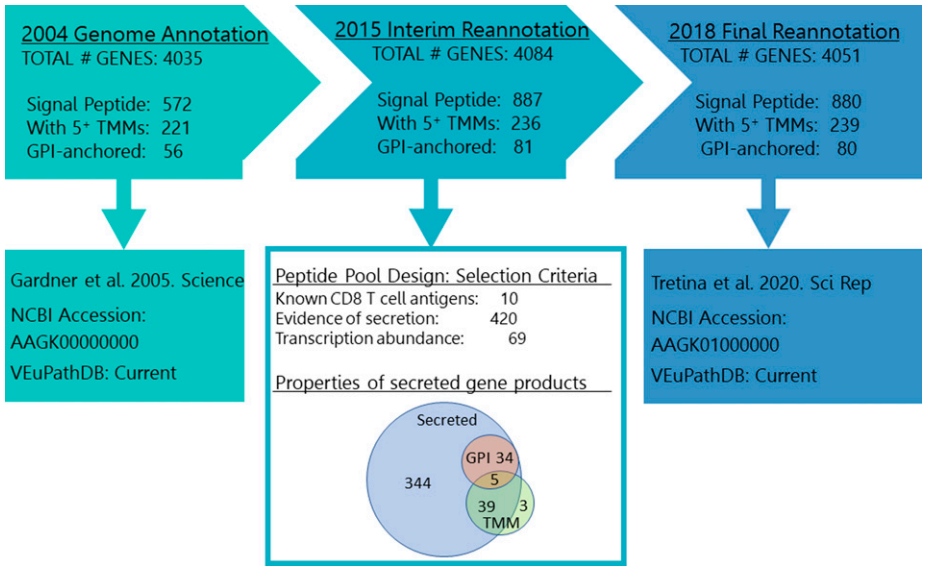

FIGURE 1. Schematic illustration of parameters used to select proteins for production of a synthetic peptide library for Ag screening, based on the predicted properties of *T. parva* gene products. GPI, proteins with a GPI anchor; Secreted, proteins possessing a signal peptide; TM, transmembrane domain.

Table I. Summary of Ags recognized by CD4 T cells from 12 cattle immunized against *T. parva*

Ag[a]	Gene Identifier[b]	CD8[c] 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			+	+			+						

[a]Previously used terminology shown in parentheses.
[b]Gene identifier is presented in format X_YYYY, where X is the nuclear chromosome number and YYYY represents the locus number.
[c]CD8 Ags Tp1, Tp2, and Tp7 reported by Graham et al. (11) and Tp9 by Hemmink et al. (12); Tp32 and Tp33 detected in recent Ag screens (N.D. MacHugh, unpublished data).

positive parasite proteins, representing 5.2% of the gene products screened. Representative results obtained with five CD4 T cell lines in the secondary screen (pools listed in Supplemental Table II) are shown in Fig. 2, and a summary of the gene products recognized by each animal is shown in Table I. CD4 T cell lines from individual animals recognized between 2 and 10 Ags (mean of 6.2). There was considerable variation between animals in the antigenic specificity of the detectable response. None of the Ags were recognized by all animals; some were detected by only 1 or 2 T cell lines, whereas responses to others were detected in up to 8 of the 12 lines assayed. Within the cell lines tested, 2 pairs of animals homozygous for DR 010:01 and DR 011:01 each shared responses to 5 Ags (5 of 11 = 45.5% and 5 of 8 = 62.5% of the Ags identified, respectively), which tended to be the strongest responses detected in these animals, confirming that specificity was influenced by MHC type. Differences in detection of responses to some Ags by the same DR genotypes may relate to differences in the overall antigenic specificity of the T cell lines, resulting in failure to detect some weak specificities and/or differences in the TCR repertoire of the animals.

Characteristics of the target Ags

As shown in Table II, which summarizes the main features of the Ags, they are encoded by genes distributed across all four chromosomes and exhibit a range of properties. They include six gene products known to induce CD8 T cell responses, four of which (Tp1, Tp2, Tp7, and Tp9) were reported previously (11, 12) and the remaining two (Tp32 and Tp33) identified in recent Ag screens (N.D. MacHugh and W.I. Morrison, unpublished data). Several members of two multimember gene families, namely the haloacid dehalogenases (HADs) and the subtelomere-encoded variable secreted proteins (SVSPs), are represented in the Ag panel. All 5 HAD proteins and 5 of the 14 SVSPs, included in the peptide library, were identified as Ags.

The panel of gene products included in the peptide library used for Ag screening was biased toward abundantly expressed genes and proteins predicted to be secreted from the parasite into the host cell cytoplasm or be otherwise exposed to the host. Reflecting this bias, a majority of the Ags recognized by CD4 T cells (19 of 26) have a predicted signal peptide; however, 7 Ags lacked a signal peptide. Only three of the Ags predicted to be secreted (Tp18, Tp19,

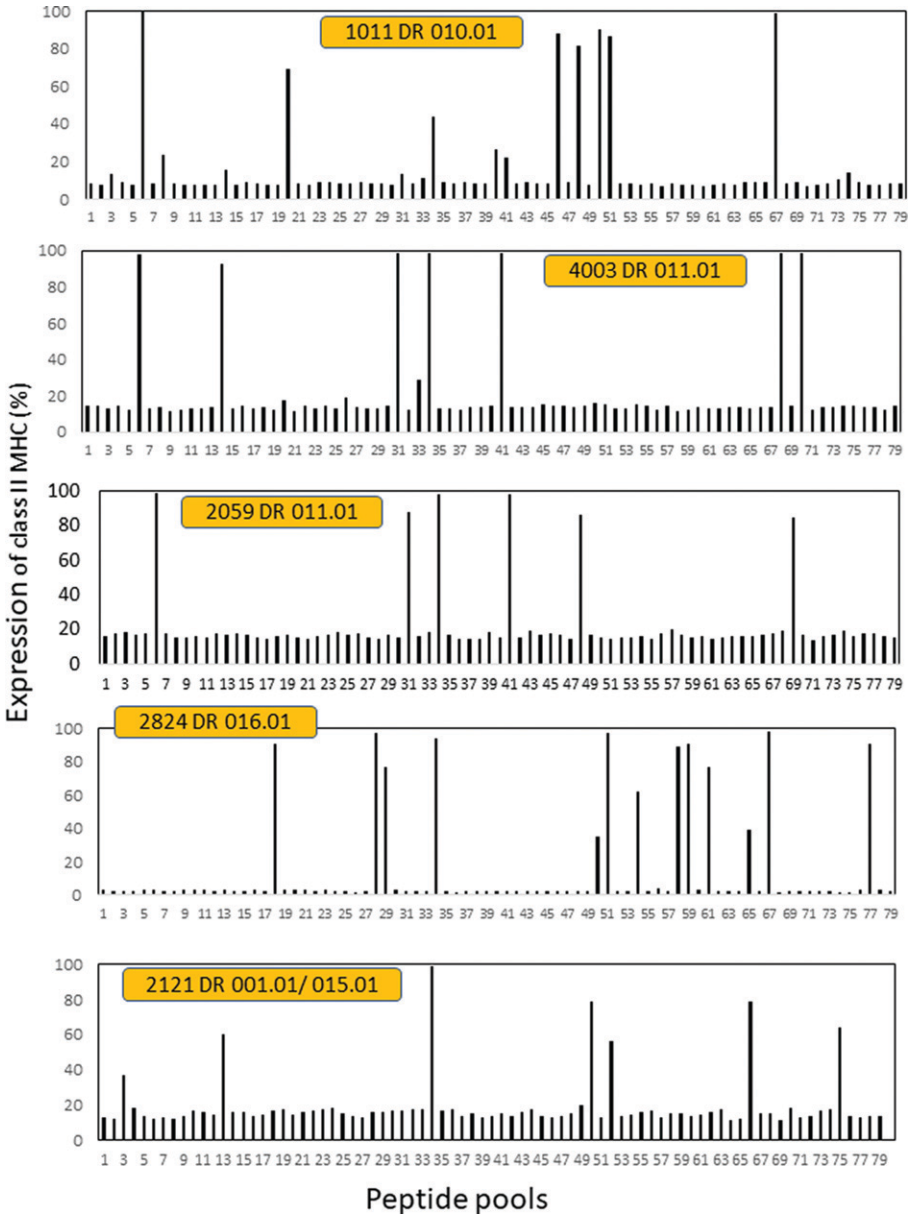

FIGURE 2. Results obtained from secondary screens of a subset of the *T. parva* peptide library with CD4 T cells from immune cattle. A set of 78 peptide pools was used, including all those detected as positive in primary screens of 514 pools, plus pools that were negative in all screens. Cells from a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

Table II. Properties of Ags recognized by CD4 T cells from cattle immunized against *T. parva*

Ag	Gene ID[a]	Protein					Gene Product[e]	Host Homologues		N[g]	π_{NS} (%)[h]	π_S (%)[i]	Expression rank[j]
		Length (aa)	Size (kD)	Sig P[b]	TM[c]	GPI[d]		Bovine	Simil. (%)[f]				
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

[a]Gene: proxy for gene identifier in the genome of the *T. parva* reference strain Muguga, where the first digit stands for chromosome number and the last four digits to the locus number in the original and the updated genome annotations [respectively, Gardner et al. (26) and Tretina et al. (46)].
[b]Sig P: presence of a signal peptide targeting the protein to the secretory pathway with high reliability, as determined with TargetP v1.1.
[c]TM: presence of one or more *trans*-membrane domains.
[d]GPI: presence of a predicted GPI anchor, as determined by GPI-SOM and/or PredGPI.
[e]Properties relating to parasite highlighted in red.
[f]Similarity: percent amino acid similarity over segment of protein aligned to host protein.
[g]Number of alleles used to estimate synonymous and nonsynonymous nucleotide diversity.
[h]Nonsynonymous nucleotide diversity: average number of nonsynonymous mutations per nonsynonymous site among *T. parva* strains infecting cattle, per 100 sites.
[i]Synonymous nucleotide diversity: average number of synonymous mutations per synonymous site among cattle-infecting *T. parva*, per 100 sites.
[j]Expression rank: expression of all *T. parva* genes in the schizont stage [Tretina et al. (38)] was ranked, with 1 being the most expressed and 4051 the least expressed, and genes assigned the corresponding rank.

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

Epitope identification and MHC restriction

Experiments to identify the target epitopes were undertaken to provide reference information on the fine specificity of the response with which to compare vaccine-induced responses. This work focused on three DR types (010:01, 011:01, and 016:01), for which the CD4 lines were derived from class II–homozygous animals. These included pairs of animals homozygous for DR 010:01 and DR 011:01. Examples of results obtained by screening five of the peptide pools are shown in Fig. 3. The 5 CD4 T cell lines examined recognized 21 of the Ags identified. Screening of individual peptides for 19 of these Ags identified 1 or occasionally 2 or 3 epitope regions within each positive pool. Where reactions to two contiguous (18-mer) peptides were detected, testing of truncated peptides allowed each epitope to be localized to within a region of 18 aa or less. The results of these assays, summarized in Table III, resulted in identification of 11–14 epitopes for each of the 3 MHC types examined. However, the HAD Ags recognized by animal 2824 contained an epitope that showed a high level of identity among all five Ags; the epitope sequences in Tp17 and Tp22 were identical, and the sequences of the other three (Tp14, Tp21, and Tp22) differed at 1, 6, and 3 of the 15-aa residues, respectively (Table III). The same epitopes were recognized by animal 598 (data not shown), which also expresses the DR 016:01 allele. Because four of these proteins were only recognized by these two DR 016:01-positive animals, it was not possible to discern whether all or only some of the Ags were responsible for inducing the CD4 T cell responses.

As expected, MHC type influenced the epitopes that were recognized. First, where Ags were recognized by animals of identical MHC types (DR 010:01 or DR 011:01), the CD4 T cell lines recognized the same epitope; conversely, within the 5 Ags recognized by 2 or 3 of the DRB3 types (Tp16, Tp9, Tp20, Tp18, and Tp15), the majority of the epitopes recognized (13 of 17) were unique to a single DRB3 type. However, in four instances, the same peptide was recognized by animals with two different class II types ($Tp9_{280-295}$ by DR 011:01 and DR 016:01; $Tp9_{292-310}$ by DR 010:01 and DR 016:01; $Tp18_{97-114}$ by DR 010:01 and DR 011:01; and $Tp20_{103-120}$ by DR 010:01 and DR 016:01).

The results obtained by testing the ability of CHO cells transfected with the DR 010:01 or DR 011:01 MHC class II alleles to

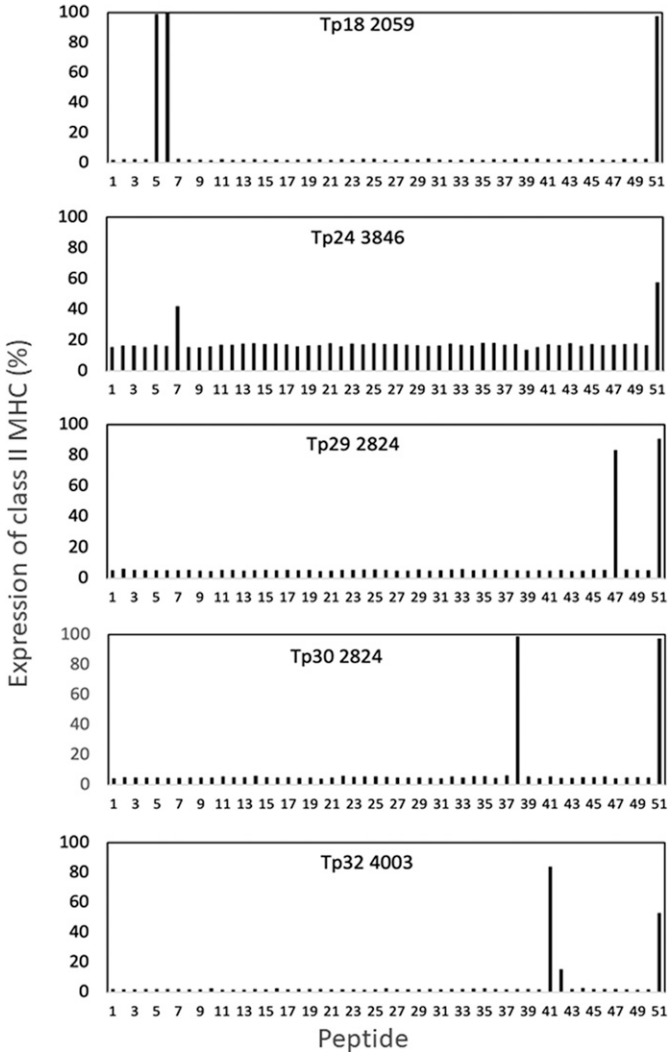

FIGURE 3. Mapping of epitopes recognized by CD4 T cells. Results are shown for responses to individual overlapping 18-mer peptides for five pools containing peptides for Tp18, Tp24, Tp29, Tp30, and Tp32, using CD4 T cells from animals 2059, 3846, 2824, 2824, and 4003, respectively. The assays used cells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

present peptides identified on the respective MHC backgrounds are shown in Fig. 4 and summarized in Table III. Thirteen of the 22 identified epitope specificities (7 on the DR 010:01 and 6 on the DR 011:01 backgrounds) were recognized when presented by CHO cells expressing the relevant DR allele. The results obtained by testing the ability of DR- and DQ-specific mAbs to inhibit epitope recognition confirmed that those epitopes that were not recognized on DR-transfected CHO cells were inhibited by the DQ-specific Ab but not by the DR-specific Ab (Fig. 5). Epitopes identified on the DR 016:01 background were tested only using the latter assay; recognition of 10 of the 11 epitopes tested was inhibited by the DR-specific Ab but not by the DQ-specific Ab. The converse result was obtained with the remaining epitope (data not shown).

Hence, overall, 22 of the unique epitopes analyzed were found to be DR-restricted and 8 were DQ-restricted. Of the four epitopes that were recognized by two different class II types, two ($Tp9_{280\text{-}295}$ and $Tp20_{103\text{-}120}$) were found to be DR-restricted and two ($Tp9_{292\text{-}310}$ and $Tp18_{97\text{-}114}$) DQ-restricted. In separate studies, the two DR-restricted epitopes were found to have a high predictive score for binding to both DR alleles (M. Nielsen, unpublished data).

Polymorphism of the identified Ags

The identification of epitopes recognized by the CD4 T cell response in individual animals, coupled with recently generated genome sequences from 17 parasite isolates obtained from cattle, allowed us to estimate the level of polymorphism across the length of the target Ags, including regions encoding the CD4 epitopes. The target Ags ranged from some of the most polymorphic in the *T. parva* genome (39), such as Tp2 and Tp9, to those that are completely conserved (Table II). They vary greatly in length and in the presence of low complexity regions or repeat regions (Fig. 6). Some of the identified epitopes were located in Ag regions with intermediate to high variability in the parasite population (e.g., Tp2, Tp9, and Tp15; Fig. 6), whereas others were in conserved regions of Ags (e.g., Tp1, Tp15, Tp16, and Tp30; Fig. 6). In a few cases, epitopes were found in regions with length variation (e.g., Tp9; Fig. 6), such that they are absent in some strains, or those adjacent to repeats (e.g., Tp9 and Tp15; Fig. 6), suggesting that protein conformation nearby may alter access to the epitopes. Overall, the CD4 T cell responses of all animals were found to recognize both conserved and polymorphic epitopes.

Specificity of CD4 T cell responses induced by prime-boost immunization with selected Ags

The context in which Ags are delivered can sometimes alter the selection of epitopes (51), with obvious consequences for the efficacy of vaccination. To determine whether *T. parva* Ags delivered in vaccine vectors induce CD4 T cell responses of similar specificities to those elicited by live parasites, four animals expressing DRB3 010:01 and/or DRB3 011:01 were immunized using a prime-

Table III. CD4 T cell epitopes[a] identified in *T. parva* Ags recognized by cattle homozygous for the DR 1001, 1101, and 1601 alleles

Ag	Gene Identifier	DR 01.001$^+$ Animal Epitope	DR 01.101$^+$ Animal Epitope	DR 01.601$^+$ Animal Epitope	MHC Restriction[b] DR	MHC Restriction[b] DQ
Tp2	1_0056		$_{76}$-LETLFGKHGLGGISKDC-$_{92}$			+
Tp16	1_0726	$_{193}$-GFLGDNMIDKSDKMPWYK-$_{210}$	$_{103}$-ITGTSQADVAMLVVPAES-$_{120}$		+	
					+	
			$_{211}$-GKILVEALDLMEPPKRPV-$_{228}$		+	
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	$_{125}$-GPYGQAGYVGQPGAVG-$_{140}$	$_{61}$-TKQDLDAKFPGMKKSK-$_{76}$	$_{281}$-DGEKVWSLEVGGDYAV-$_{296}$		+
		$_{292}$-GDYAVKVLVFPIGFKEKTI-$_{310}$	$_{280}$-YDGEKVWSLEVGGDYA-$_{295}$	$_{292}$-GDYAVKVLVFPIGFKEKTI-$_{310}$		+
			$_{307}$-EKTIEITFIGGEKEIY-$_{322}$		+	
						+
Tp13	3_0655	$_{26}$-GRVSNYVTYAKKLLSNGL-$_{43}$			+	
					+	
Tp1	3_0849		$_{487}$-SIVNVYGKNDEPLSYAPS-$_{504}$			+
Tp20	3_0861	$_{103}$-QEILYYKWEKHGFVKETY-$_{120}$		$_{103}$-QEILYYKWEKHGFVKETY-$_{120}$	+	
		$_{577}$-PLSGYHVRYVNYGKVIMW-$_{594}$		$_{451}$-FNKFDMLHDGVYYSSPVP-$_{468}$	+	
				$_{1351}$-CKANNPVVYIKAGDKTVW-$_{1368}$	+	
					+	
Tp18	4_0051	$_{97}$-QQGPDTPQPIQEPSGPVQ-$_{114}$	$_{97}$-QQGPDTPQPIQEPSGPVQ-$_{114}$			+
Tp19	4_0437		$_{835}$-KSFDDLTTVELAPEPKAS-$_{852}$		+	
Tp29	4_0683			$_{499}$-QIEVTFNIDTNGILSVTA-$_{516}$	+	
Tp30	4_0752			$_{133}$-NCGRGVFMAAHNNRTYCG-$_{150}$	+	
Tp15	4_0916	$_{105}$-GTYQHYGPPVFPPQPE-$_{116}$	$_{517}$-RNQVVWIKTASEGFPSSM-$_{534}$	$_{385}$-IEMTEKEYKIIVDSRF-$_{400}$	+	
		$_{157}$-GIQYVPYQTLQIPQPQ-$_{172}$			+	
					+	
						+

[a]The location of the epitopes was determined by screening overlapping 18-mer peptides (overlapping by 12 residues) for the respective positive Ag, using an IFN-γ release assay as described by Hart et al. (31). In some cases, further truncated peptides were tested.

[b]The class II restriction of T cells specific for the epitopes was determined using two assays, first by testing recognition of the peptides preincubated with CHO cells expressing the 1001 or 1101 class II DR alleles and, second by testing the ability of mAbs specific for bovine DR or DQ (IL-A21 and CC158, respectively) to inhibit recognition of the peptides.

NT, not tested.

boost protocol, with the Tp9 and Tp15 Ags expressed in recombinant adenovirus and vaccinia (MVA) vectors. CD4 T cell lines generated from all four animals, by stimulation of T cells with autologous *T. parva*–infected cells, recognized Tp9 and Tp15 (Table IV). Eight epitopes had been identified in these two Ags during Ag screening with T cell lines expressing DRB3 010:01 or DRB3 011:01. Epitope mapping of the CD4 response in one of the viral vector–immunized animals, which expressed both DRB3 010:01 and DRB3 011:01, revealed significant responses of variable magnitude to six epitopes, four in Tp9 and two in Tp15. These epitopes were identical to those recognized by *T. parva*–immune animals (Table V), confirming the capacity of the vectored Ags to induce CD4 T cell responses to epitope specificities displayed on infected cells.

The same four animals were concurrently immunized with recombinant adenovirus/MVA viruses expressing a third Ag (Tp10) (12) known to induce CD8 T cell responses but not identified as a CD4 T cell target Ag by screening in the current study. All four animals generated a CD4 T cell response to Tp10, detectable by stimulation in vitro with *T. parva*–infected cells (Table IV). Hence, this Ag is presented by parasitized cells and can be immunogenic (i.e., without the context of natural infection), even though it was not identified as a CD4 T cell Ag in the peptide screens.

Data on CD8 responses to these Ags were also obtained from three of the animals. Significant responses were detected in all three animals for Tp9 and in two animals for Tp10, but none of the animals responded to Tp15 (Table IV).

Discussion

The evidence that CD8 T cells play a key role in immunity to *T. parva* has led to efforts to develop a vaccine using CD8 T cell target Ags. CD4 T cell responses are also likely to be required to provide help for efficient induction and recall of CD8 T cell responses. Although infection with *T. parva* induces vigorous CD4 T cell responses against infected cells, there is a paucity of information on their specificity. The results of the current study demonstrate that, in contrast to the profound immunodominance of CD8 T cell responses to *T. parva* in immune cattle by which a large proportion of the response in individual animals is focused on one or two Ags (10), CD4 T cell responses recognize multiple Ags, including both polymorphic and conserved Ags. Ag screens using CD4 T cells from 12 immune cattle of different MHC class II types identified 26 Ags, with up to 11 antigenic specificities detected in a single animal. As would be expected, there was variation between animals in the Ags that were recognized, with no single antigenic specificity detected in all animals.

Despite the multiple Ags identified in this study, the findings almost certainly underestimate the diversity of antigenic specificities presented by *T. parva*–infected cells. Because the peptide library represented only ~12.5% of *T. parva* genes, almost all of which are transcribed by the schizont stage of the parasite (46), the remaining proteome is likely to include additional unidentified Ags. Moreover, the assay used for Ag screening may well fail to detect T cell specificities present at low frequency. The finding that immunization of

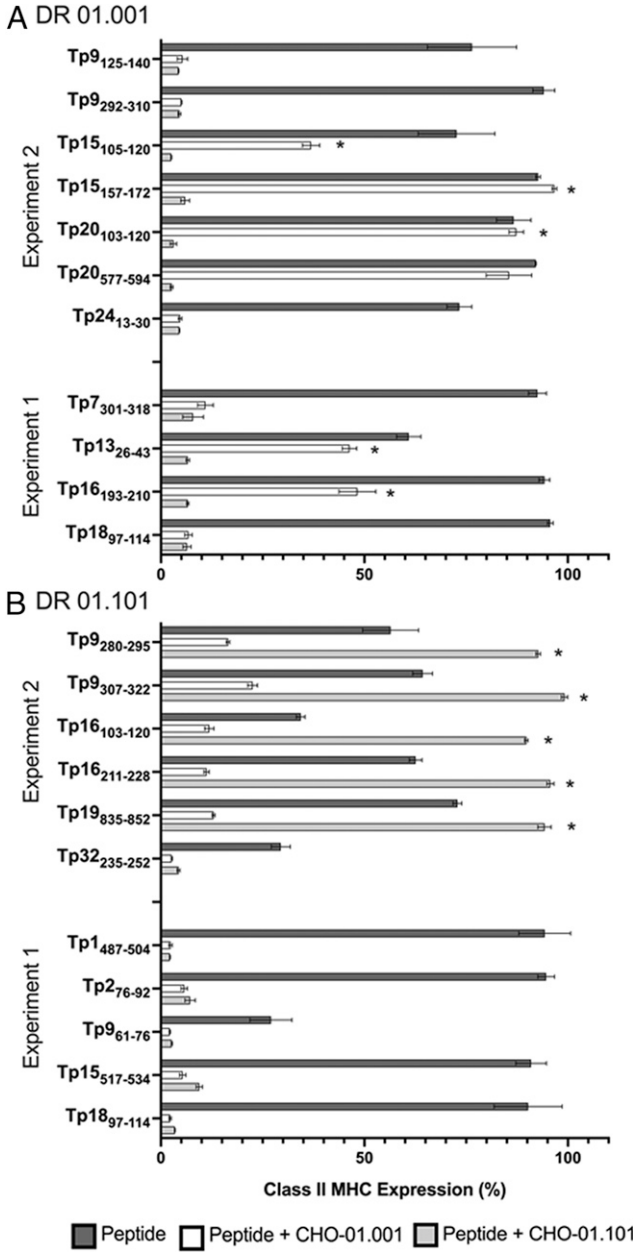

FIGURE 4. MHC class II restriction of CD4 T cells specific for *T. parva* epitopes. Presentation of individual epitopes by MHC class II DR was examined by measuring responses to CHO cells transfected with the DRB3 010:01 or DRB3 011:01 alleles, which had been preincubated with peptide and washed prior to incubation with CD4 T cells. CD4 T cell lines from animals homozygous for DRB3 010:01 or DRB3 011:01, were assayed in two experiments for each (**A** and **B**). The assays used cells from a *T. parva*–stimulated CD4 T cell line from each animal, tested in duplicate wells. Reactivity of the CD4 T cells was assayed by testing the ability of supernatants of stimulated T cells to upregulate expression of surface MHC class II on MDBK cells, as a measure of IFN-γ release. Results are shown for responses to peptide added directly to CD4 T cells and autopresented (positive control), responses against peptide preincubated with CHO cells expressing an irrelevant BoLA-DR molecule (negative control) and responses against peptides preincubated with CHO cells expressing the relevant BoLA-DR molecule. $*p < 0.05$ compared with responses to cells expressing the irrelevant DR.

calves with viral vectors expressing the Tp10 CD8 Ag, which was not detected by the Ag screens, induced CD4 responses reactive with *T. parva*–infected cells, clearly illustrates the presence of additional immunogenic antigenic specificities on infected cells.

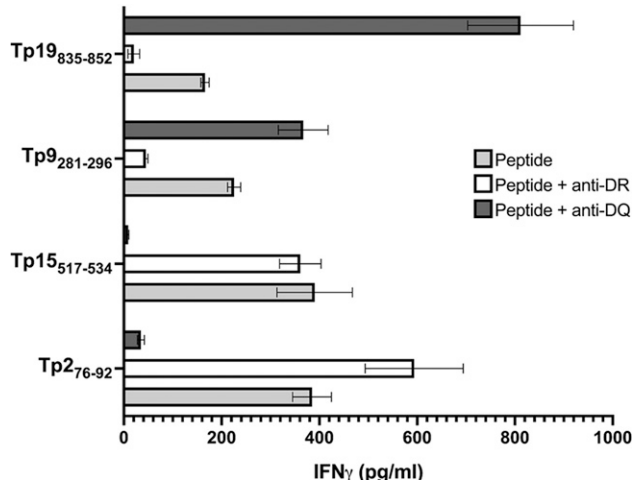

FIGURE 5. MHC class II DR or DQ restriction of CD4 T cells specific for *T. parva* epitopes. Inhibition of epitope recognition by mAbs specific for bovine DR (IL-A21) and DQ (CC158) was examined by measuring IFN-γ release in supernatants of peptide-stimulated CD4 T cells using a specific ELISA. Cells from a *T. parva*–stimulated CD4 T cell line from each animal were tested in duplicate wells. Results are shown for two DR-restricted ($Tp16_{103\text{-}120}$ and $Tp19_{835\text{-}852}$) and two DQ-restricted epitopes ($Tp2_{76\text{-}92}$ and $Tp15_{517\text{-}534}$). Responses to peptide in the absence of added Ab are shown in gray. For each epitope, inhibition by the respective Ab was observed compared with the noninhibitory Ab. $p < 0.01$.

The strategy employed in the current study for Ag screening was based on knowledge of the biology of the parasite and how parasite Ags are likely to be recognized by CD4 T cells in vivo. Unlike many other apicomplexan parasites, *T. parva* is found free within the host cell cytoplasm, having escaped from the endocytic vacuole shortly after initial cell invasion by the sporozoite (52). The parasite establishes an intimate relationship with the host cell, which results in activation and transformation of infected host cells and inhibition of apoptosis (53). Synchronous division of the parasite and host cell (54, 55) results in clonal expansion of the infected cell population, thus ensuring that the parasites are retained intracellularly throughout this stage of development. The transformed state of the infected cells also results in expression of surface DR and DQ MHC class II proteins, as well as costimulatory proteins such as CD80, CD86, and CD40 (W.I. Morrison, unpublished data). These properties of infected cells favor endogenous routes of processing of parasite Ags and allow direct recognition of infected cells by parasite-specific CD4 T cells, in the absence of added APCs. Hence, the current study used CD4 T cell lines generated from immune cattle by stimulation in vitro with intact parasitized cells for Ag screening. Holstein cattle, which are highly susceptible to *T. parva* and are extensively used for milk production in endemic areas of Africa, were used in the study. The MHC class II types of the animals included the most frequently expressed DR alleles in Holsteins; a recent study of 331 Holstein cattle identified 15 DR alleles, 10 of which were represented in the current study and included the 7 most frequently detected alleles (56).

T. parva schizont Ags targeted by CD4 T cells on infected cells must access the cytoplasm of the infected cells, prior to entering MHC class II processing pathways. Consequently, proteins secreted from the parasite or released from the parasite by other mechanisms represent likely candidate Ags. Studies of Ag processing of a number of human and murine intracellular pathogens indicate that cytoplasmic proteins can be transferred into the endosomal pathway for class II processing either following autophagy within the cytoplasm

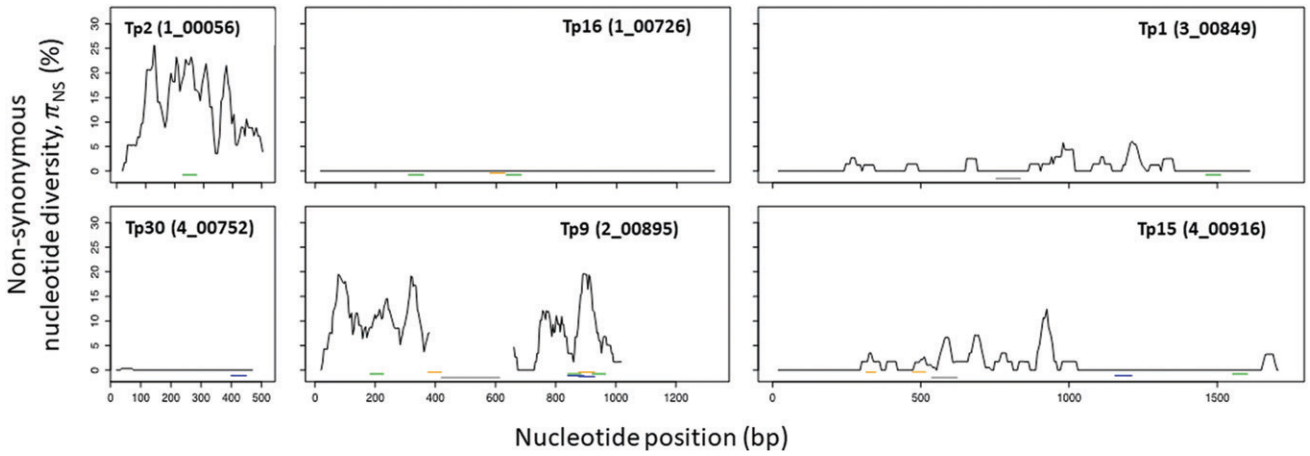

FIGURE 6. Amino acid sequence variation across coding sequence and location of epitopes: Average pairwise difference per nonsynonymous site between sequences (π_{NS}) is shown for six representative Ags. π_{NS} was calculated using a sliding window of 30 sites, with a 5-site step. The π_{NS} plot is interrupted in regions of the alignment with indels between alleles. Repeat regions are shown in gray below the plot. The location of epitopes shown in Table III is marked by MHC genotype in yellow (DR 10:01), green (DR 11:01), and blue (DR 16:01).

or as peptides generated by degradation of parasite proteins within the proteasome (57, 58). The involvement of the latter pathway has been demonstrated for some CD4 T cell responses to influenza virus–infected cells (59–61). In preliminary studies, we have found that recognition of the Tp9 Ag on infected cells by specific CD4 T cell clones is inhibited by preincubation of the infected cells with the proteasome inhibitor epoxomicin (A. Aguada and W.I. Morrison, unpublished data), suggesting that this endogenous pathway is involved in processing *T. parva* Ags.

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

Only 6 of the 14 CD8 Ags identified to date (Tp1, Tp2, Tp7, Tp9, Tp32, and Tp33) were also identified as CD4 T cell Ags. Previous studies have shown that Tp1 and Tp2 are highly dominant CD8 Ags in class I A18 and A10 animals, respectively, yet these Ags were not recognized, to a detectable level, by CD4 T cells from animals of these class I genotypes. Thus, the CD4 and CD8 T cell responses in these animals have different dominant antigenic specificities. A recent study of prime-boost immunization of class I A18$^+$ animals with Tp1 expressed in adeno and vaccinia virus vectors observed Tp1-specific CD4 responses during the active response to the vaccinia boost. However, because the T cell responses were monitored using Tp1 Ag added to APCs, the findings did not confirm recognition of the Ag presented by infected cells. Nevertheless, the findings suggest that, as with our results with vector-induced responses to Tp10, vaccination with defined Ags may elicit specificities that are not readily detected during responses to infection.

All three proteins previously shown to induce Ab responses to the schizont stage of the parasite in infected animals (PIM, p104, and p150; now, respectively, Tp18, Tp19, and Tp20) (47, 62, 63) were identified as CD4 Ags, but only in a subset of the animals. Notably, CD4 T cell responses to the Tp18 Ag (PIM), which is an abundant schizont protein and induces highly dominant Ab responses in all immune animals (47), were detected in only two of the animals examined. This implies that additional exogenous routes of processing of some Ags released from infected cells may be involved in vivo in generating CD4 T cell responses, due to more efficient display of Ag on APCs. Although a majority of the identified Ags (19 of 26) had predicted signal peptides, this appeared to reflect the composition of the peptide library, 84% of which was generated from proteins with a predicted peptide sequence.

Table IV. CD4 and CD8 T cell responses of four animals immunized with recombinant adenovirus and vaccinia viruses expressing the Tp9, Tp10, and Tp15 *T. parva* Ags

	T Cell Lines and MHC Genotypes[b]							
	2750 MHC I—A10/A14 DR-01.001/01.101		2733 MHC I—A11/A14 DR-01.101/01.401		2751 MHC I—A14/A15 DR-01.101/01.401		2758 MHC I—A10/A11 DR-01.001/01.101	
Antigens[a]	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)

[a]The Ags consisted of pools of overlapping 18-mer synthetic peptides for each Ag. Controls represent responses to a peptide pool not recognized on these MHC backgrounds.

[b]Responses were measured by testing the ability of supernatants from Ag-stimulated T cells to upregulate expression of surface MHC class II on MDBK cells. Results are expressed as the % of MHC class II–positive cells, showing the mean (SD) for duplicates of each assay.

*$p < 0.05$, **$p < 0.01$.

MHC I, MHC class I; NT, not tested.

Table V. Response to known Tp9 and Tp15 epitopes of CD4 T cells from an animal (2750) vaccinated with the Tp9 and Tp15 Ags expressed in recombinant adenovirus and vaccinia vaccine vectors

	Responses of CD4 T Cell Lines (MHC DR Type)[a]		
Antigen	3846 (01.001/01.001)	4003 (01.101/01.101)	2750 (01.001/01.101)
Control[b]	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)*

[a]The 3846 and 4003 CD4 T cell lines were from *T. parva*–immune MHC-homozygous animals, which were used for Ag identification. Responses were measured by testing the ability supernatants from peptide-stimulated T cells to upregulate expression of surface MHC class II on MDBK cells. Results are expressed as the percentage of MHC class II–positive cells, showing the mean (SD) for duplicates of each assay.

[b]Controls represent responses to a peptide not recognized on either of these MHC backgrounds.

*$p < 0.05$, **$p < 0.01$.

Abundance of transcription of the Ag-encoding genes was highly variable: although half of the Ags (13 of 26) were encoded by the top 10% most abundantly transcribed genes, and others were in the lower 50%. Because transcript abundance was used to select proteins that lacked a signal sequence, the Ags identified within this subset were encoded by some of the most abundantly transcribed genes (10 of 11 ranked in the top 100). Although neither of these parameters on their own appeared reliable for predicting CD4 cell target Ags, the large number of Ags detected in the current study suggest that both properties may have been useful for Ag identification. However, screening of a large unbiased panel of parasite gene products would be required to address this question.

Although the identified CD4 Ags included proteins with diverse properties, several members of the HAD and SVSP families of proteins were identified. All five HAD proteins included in the peptide library were identified as Ags. However, epitope mapping revealed a CD4 T cell epitope that was highly conserved between the five HAD proteins and, because this was the only epitope identified in four of the Ags, it was unclear whether only some or all of these Ags were responsible for inducing the T cell responses. The HAD family of proteins are phosphatases found in a wide range of prokaryotic and eukaryotic organisms, identifiable by the presence of conserved sequence motifs (64). *Plasmodium falciparum* has 23 HAD-encoding genes, 2 of which have been implicated as targets for the experimental therapeutic compound fosmidomycin, which targets the methyl-erythritol phosphate pathway of the apicomplast organelle (65). However, unlike the *T. parva* HAD proteins identified as Ags, these *Plasmodium* proteins do not possess signal peptides and hence the former are likely to have different biological functions, potentially involving interaction with host proteins. The SVSP family, which has only been described in *Theileria*, consists of 85 members encoded by genes located in the telomeric regions of all 4 nuclear chromosomes (66, 67), 14 of which were included in the peptide library. They encode a short, conserved N-terminal region including a signal peptide, followed by a QP-rich central region and a conserved C terminus. Some contain a functional nuclear localization signal and some are expressed by only a proportion of cells in cultures of infected cells. However, as with the HAD proteins, the functions of SVSPs remain unclear. Further studies are required to explore whether additional members of the SVSP family, not included in the peptide library, are recognized by parasite-specific T cells.

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

With regard to vaccination, the findings have provided valuable information on which to base selection of Ags suitable for vaccine development. As discussed earlier, there is evidence that parasite-specific CD4 T cell responses are required for efficient activation of memory CD8 T cells, and in vitro studies suggest that such help requires Ags to be displayed on the same APCs for recognition by CD4 and CD8 T cells (19). This is clearly the case for *T. parva*–infected cells, which express both MHC class I and class II proteins on their cell surface, allowing activation of both CD4 and CD8 T cell responses following infection. The evidence that both CD8 and CD4 Ags undergo intracellular (endogenous) routes of Ag processing in infected cells implies that the same Ag delivery systems can be used in a subunit vaccine to generate both CD4 and CD8 T cell responses. In this regard, it is significant that immunization of calves with recombinant adeno and vaccinia virus vectors expressing two of the identified Ags were found to induce both CD4 and CD8 T cell responses reactive with parasitized cells and that the epitopes recognized by the CD4 T cells were identical to those induced by infection with *T. parva.* Although induction of CD8 T cell responses by Ags expressed in viral vectors could theoretically use help provided by CD4 T cells specific for the vector viruses, efficient recall of such responses following parasite challenge will require vaccine-induced CD4 T cells responses against epitopes presented by *T. parva*–infected cells. The present study has shown that *T. parva*–infected cells display CD4 epitopes from a range of different parasite proteins. Data on polymorphism will permit selection of Ags with conserved amino acid sequences to avoid strain specificity of CD4 T cell responses. Further studies

are required to determine the capacity of candidate Ags to induce CD4 T cell responses in animals of different MHC genotypes when delivered in suitable vaccine vectors. Although responses of immune animals to most of the Ags in the current study were only detected in certain MHC genotypes, it is possible that immunization with vaccine vectors will reveal immunogenicity in a wider range of genotypes. Similar investigations are ongoing to identify CD8 Ags with these properties. If different Ags prove optimal for induction of CD4 and CD8 T cell responses, it will be important to determine whether they can be delivered separately or need to be incorporated into the same vaccine vector for optimal results.

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

Acknowledgments

We thank Professor Sarah Gilbert, The Jenner Institute, Oxford University, for producing recombinant vaccinia and adeno viruses and JPT Peptide Technologies GmbH for advice on the peptide library. We also thank Christina Vrettou for expert technical assistance. We dedicate this publication to Dr. Niall D. MacHugh, who initiated some of the early experiments reported in this study but unfortunately died in 2017.

Disclosures

The authors declare no conflict of interest.

References

1. Irvin, A. D., and W. I. Morrison. 1987. Immunopathology, immunology and immunoprophylaxis of Theileria infections. In: *Immune Responses in Parasitic Infections: Immunology, Immunopathology and Immunoprophylaxis, Vol. III. Protozoa*. L. Soulsby, ed. CRC Press, Baton Rouge, Florida, pp. 223–274.
2. Radley, D. E., C. G. D. Brown, M. J. Burridge, M. P. Cunningham, I. M. Kirimi, R. E. Purnell, and A. S. Young. 1975. East Coast fever: 1. Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five theilerial strains. *Vet. Parasitol.* 1: 35–41.
3. Radley, D. E., C. G. D. Brown, M. P. Cunningham, C. D. Kimber, F. L. Musisi, R. C. Payne, R. E. Purnell, S. M. Stagg, and A. S. Young. 1975. East Coast fever: 3. Chemoprophylactic immunization of cattle using oxytetracycline and a combination of theilerial strains. *Vet. Parasitol.* 1: 51–60.
4. Norling, M., R. P. Bishop, R. Pelle, W. Qi, S. Henson, E. F. Drábek, K. Tretina, D. Odongo, S. Mwaura, T. Njoroge, et al. 2015. The genomes of three stocks comprising the most widely utilized live sporozoite *Theileria parva* vaccine exhibit very different degrees and patterns of sequence divergence. *BMC Genomics* 16: 729.
5. Di Giulio, G., G. Lynen, S. Morzaria, C. Oura, and R. Bishop. 2009. Live immunization against East Coast fever–current status. *Trends Parasitol.* 25: 85–92.
6. Nene, V., and W. I. Morrison. 2016. Approaches to vaccination against *Theileria parva* and *Theileria annulata*. *Parasite Immunol.* 38: 724–734.
7. Morrison, W. I., T. Connelley, J. D. Hemmink, and N. D. MacHugh. 2015. Understanding the basis of parasite strain-restricted immunity to *Theileria parva*. *Annu. Rev. Anim. Biosci.* 3: 397–418.
8. McKeever, D. J., E. L. Taracha, E. L. Innes, N. D. MacHugh, E. Awino, B. M. Goddeeris, and W. I. Morrison. 1994. Adoptive transfer of immunity to *Theileria parva* in the CD8+ fraction of responding efferent lymph. *Proc. Natl. Acad. Sci. USA* 91: 1959–1963.
9. Taracha, E. L., B. M. Goddeeris, A. J. Teale, S. J. Kemp, and W. I. Morrison. 1995. Parasite strain specificity of bovine cytotoxic T cell responses to *Theileria parva* is determined primarily by immunodominance. *J. Immunol.* 155: 4854–4860.
10. MacHugh, N. D., T. Connelley, S. P. Graham, R. Pelle, P. Formisano, E. L. Taracha, S. A. Ellis, D. J. McKeever, A. Burrells, and W. I. Morrison. 2009. CD8+ T-cell responses to *Theileria parva* are preferentially directed to a single dominant antigen: Implications for parasite strain-specific immunity. *Eur. J. Immunol.* 39: 2459–2469.
11. Graham, S. P., R. Pellé, Y. Honda, D. M. Mwangi, N. J. Tonukari, M. Yamage, E. J. Glew, E. P. de Villiers, T. Shah, R. Bishop, et al. 2006. *Theileria parva* candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 103: 3286–3291.
12. Hemmink, J. D., W. Weir, N. D. MacHugh, S. P. Graham, E. Patel, E. Paxton, B. Shiels, P. G. Toye, W. I. Morrison, and R. Pelle. 2016. Limited genetic and antigenic diversity within parasite isolates used in a live vaccine against *Theileria parva*. *Int. J. Parasitol.* 46: 495–506.
13. Svitek, N., R. Saya, E. Awino, S. Munyao, R. Muriuki, T. Njoroge, R. Pellé, N. Ndiwa, J. Poole, S. Gilbert, et al. 2018. An Ad/MVA vectored *Theileria parva* antigen induces schizont-specific $CD8^+$ central memory T cells and confers partial protection against a lethal challenge. *NPJ Vaccines* 3: 35.
14. Swain, S. L., K. K. McKinstry, and T. M. Strutt. 2012. Expanding roles for $CD4^+$ T cells in immunity to viruses. *Nat. Rev. Immunol.* 12: 136–148.
15. Tsuji, M., and F. Zavala. 2003. T cells as mediators of protective immunity against liver stages of *Plasmodium*. *Trends Parasitol.* 19: 88–93.
16. Tse, S.-W., A. J. Radtke, and F. Zavala. 2011. Induction and maintenance of protective CD8+ T cells against malaria liver stages: implications for vaccine development. *Mem. Inst. Oswaldo Cruz* 106(Suppl. 1): 172–178.
17. Crispe, I. N. 2014. APC licensing and CD4+T cell help in liver-stage malaria. *Front. Microbiol.* 5: 617.
18. Baldwin, C. L., K. P. Iams, W. C. Brown, and D. J. Grab. 1992. Theileria parva: CD4+ helper and cytotoxic T-cell clones react with a schizont-derived antigen associated with the surface of Theileria parva-infected lymphocytes. *Exp. Parasitol.* 75: 19–30.
19. Taracha, E. L., E. Awino, and D. J. McKeever. 1997. T helper requirements in *Theileria parva*-immune and -naïve CTL precursors. *J. Immunol.* 159: 4539–4545.
20. Brown, W. C., C. Sugimoto, and D. J. Grab. 1989. *Theileria parva*: bovine helper T cell clones specific for both infected lymphocytes and schizont membrane antigens. *Exp. Parasitol.* 69: 234–248.
21. Brown, W. C., J. D. Lonsdale-Eccles, J. C. DeMartini, and D. J. Grab. 1990. Recognition of soluble Theileria parva antigen by bovine helper T cell clones: characterization and partial purification of the antigen. *J. Immunol.* 144: 271–277.
22. Grab, D. J., C. L. Baldwin, W. C. Brown, E. A. Innes, J. D. Lonsdale-Eccles, and Y. Verjee. 1992. Immune CD4+ T cells specific for Theileria parva-infected lymphocytes recognize a 24-kilodalton protein. *Infect. Immun.* 60: 3892–3896.
23. Ellis, S. A., W. I. Morrison, N. D. MacHugh, J. Birch, A. Burrells, and M. J. Stear. 2005. Serological and molecular diversity in the cattle MHC class I region. *Immunogenetics* 57: 601–606.
24. Ellis, S. A., K. A. Staines, M. J. Stear, E. J. Hensen, and W. I. Morrison. 1998. DNA typing for BoLA class I using sequence-specific primers (PCR-SSP). *Eur. J. Immunogenet.* 25: 365–370.
25. Baxter, R., N. Hastings, A. Law, and E. J. Glass. 2008. A rapid and robust sequence-based genotyping method for BoLA-DRB3 alleles in large numbers of heterozygous cattle. *Anim. Genet.* 39: 561–563.
26. Gardner, M. J., R. Bishop, T. Shah, E. P. de Villiers, J. M. Carlton, N. Hall, Q. Ren, I. T. Paulsen, A. Pain, M. Berriman, et al. 2005. Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. *Science* 309: 134–137.
27. Brown, C. G., D. A. Stagg, R. E. Purnell, G. K. Kanhai, and R. C. Payne. 1973. Letter: Infection and transformation of bovine lymphoid cells in vitro by infective particles of Theileria parva. *Nature* 245: 101–103.
28. Goddeeris, B. M., and W. I. Morrison. 1988. Techniques for the generation, cloning and characterisation of bovine cytotoxic T cells specific for the protozoan *Theileria parva*. *J. Tissue Cult. Methods* 11: 101–110.
29. Ballingall, K. T., D. M. Mwangi, N. D. MacHugh, E. L. Taracha, P. Totte, and D. J. McKeever I. 2000. A highly sensitive, non-radioactive assay for T cell activation in cattle: applications in screening for antigens recognised by CD4(+) and CD8(+) T cells. *J. Immunol. Methods* 239: 85–93.
30. Ryan, M. D., and J. Drew. 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. *EMBO J.* 13: 928–933.
31. Hart, J., N. D. MacHugh, T. Sheldrake, M. Nielsen, and W. I. Morrison. 2017. Identification of immediate early gene products of bovine herpes virus 1 (BHV-1) as dominant antigens recognized by CD8 T cells in immune cattle. *J. Gen. Virol.* 98: 1843–1854.
32. Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10: 1–6.
33. Emanuelsson, O., H. Nielsen, S. Brunak, and G. von Heijne. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300: 1005–1016.
34. Fankhauser, N., and P. Mäser. 2005. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. *Bioinformatics* 21: 1846–1852.
35. Pierleoni, A., P. L. Martelli, and R. Casadio. 2008. PredGPI: a GPI-anchor predictor. *BMC Bioinformatics* 9: 392.
36. Sonnhammer, E. L., G. von Heijne, and A. Krogh. 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 6: 175–182.
37. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305: 567–580.
38. Tretina, K., R. Pelle, and J. C. Silva. 2016. Cis regulatory motifs and antisense transcriptional control in the apicomplexan Theileria parva. *BMC Genomics* 17: 128.
39. Palmateer, N., K. Tretina, J. Orvis, I. Olukemi, J. Crabtree, E. Drabék, R. Pelle, E. Awino, H. T. Gotia, J. B. Munro, et al. 2020. Capture-based enrichment of *Theileria parva* genomic DNA enables whole genome assembly of a buffalo-

derived strain and reveals distinct, host-associated parasite subspecies. *PLoS Negl. Trop. Dis.* 14: e0008781.
40. Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32: 1792–1797.
41. Maddison, W. P., and D. R. Maddison. 2019. Mesquite: a modular system for evolutionary analysis. Version 3.61. Available at: https://www.mesquiteproject.org. Accessed: February 14, 2021.
42. Rozas, J., A. Ferrer-Mata, J. C. Sánchez-DelBarrio, S. Guirao-Rico, P. Librado, S. E. Ramos-Onsins, and A. Sánchez-Gracia. 2017. DnaSP 6: DNA sequence polymorphism analysis of large datasets. *Mol. Biol. Evol.* 34: 3299–3302.
43. Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27: 573–580.
44. R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org. Accessed: March 1, 2021.
45. Almagro Armenteros, J. J., M. Salvatore, O. Emanuelsson, O. Winther, G. von Heijne, A. Elofsson, and H. Nielsen. 2019. Detecting sequence signals in targeting peptides using deep learning. *Life Sci. Alliance* 2: e201900429.
46. Tretina, K., R. Pelle, J. Orvis, H. T. Gotia, O. O. Ifeonu, P. Kumari, N. C. Palmateer, S. B. A. Iqbal, L. M. Fry, V. M. Nene, et al. 2020. Re-annotation of the *Theileria parva* genome refines 53% of the proteome and uncovers essential components of N-glycosylation, a conserved pathway in many organisms. *BMC Genomics* 21: 279.
47. Toye, P. G., B. M. Goddeeris, K. Iams, A. J. Musoke, and W. I. Morrison. 1991. Characterization of a polymorphic immunodominant molecule in sporozoites and schizonts of Theileria parva. *Parasite Immunol.* 13: 49–62.
48. Xue, G., C. von Schubert, P. Hermann, M. Peyer, R. Maushagen, J. Schmuckli-Maurer, P. Bütikofer, G. Langsley, and D. A. E. Dobbelaere. 2010. Characterisation of gp34, a GPI-anchored protein expressed by schizonts of Theileria parva and T. annulata. *Mol. Biochem. Parasitol.* 172: 113–120.
49. Woods, K. L., R. Theiler, M. Mühlemann, A. Segiser, S. Huber, H. R. Ansari, A. Pain, and D. A. E. Dobbelaere. 2013. Recruitment of EB1, a master regulator of microtubule dynamics, to the surface of the *Theileria annulata* schizont. *PLoS Pathog.* 9: e1003346.
50. Huber, S., R. Theiler, D. de Quervain, O. Wiens, T. Karangenc, V. Heussler, D. Dobbelaere, and K. Woods. 2017. The microtubule-stabilising protein CLASP-1 associates with the *Theileria annulata* schizont surface via its kinetochore-binding domain. *MSphere* 2: e00215–e00217.
51. Veerappan Ganesan, A. P., and L. C. Eisenlohr. 2017. The elucidation of non-classical MHC class II antigen processing through the study of viral antigens. *Curr. Opin. Virol.* 22: 71–76.
52. Fawcett, D. W., S. Doxsey, D. A. Stagg, and A. S. Young. 1982. The entry of sporozoites of Theileria parva into bovine lymphocytes in vitro. Electron microscopic observations. *Eur. J. Cell Biol.* 27: 10–21.
53. Dobbelaere, D. A., and S. Rottenberg. 2003. *Theileria*-induced leukocyte transformation. *Curr. Opin. Microbiol.* 6: 377–382.
54. Hulliger, L., K. H. Wilde, C. G. D. Brown, and L. Turner. 1964. Mode of multiplication of *Theileria i*n cultures of bovine lymphocytic cells. *Nature* 203: 728–730.
55. von Schubert, C., G. Xue, J. Schmuckli-Maurer, K. L. Woods, E. A. Nigg, and D. A. Dobbelaere. 2010. The transforming parasite *Theileria* co-opts host cell mitotic and central spindles to persist in continuously dividing cells. *PLoS Biol.* 8: e1000499.
56. Vasoya, D., P. S. Oliveira, L. A. Muriel, T. Tzelos, C. Vrettou, W. I. Morrison, I. K. F. de Miranda Santos, and T. Connelley. 2021. High throughput analysis of MHC-I and MHC-DR diversity of Brazilian cattle populations. *HLA* 98: 93–113.
57. Gannage, M., and C. Münz. 2010. MHC presentation via autophagy and how viruses escape from it. *Semin. Immunopathol.* 32: 373–381.
58. Neefjes, J., M. L. M. Jongsma, P. Paul, and O. Bakke. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11: 823–836.
59. Tewari, M. K., G. Sinnathamby, D. Rajagopal, and L. C. Eisenlohr. 2005. A cytosolic pathway for MHC class II-restricted antigen processing that is proteasome and TAP dependent. [Published erratum appears in 2005 *Nat. Immunol.* 6: 420.] *Nat. Immunol.* 6: 287–294.
60. Eisenlohr, L. C., N. Luckashenak, S. Apcher, M. A. Miller, and G. Sinnathamby. 2011. Beyond the classical: influenza virus and the elucidation of alternative MHC class II-restricted antigen processing pathways. *Immunol. Res.* 51: 237–248.
61. Miller, M. A., A. P. V. Ganesan, N. Luckashenak, M. Mendonca, and L. C. Eisenlohr. 2015. Endogenous antigen processing drives the primary CD4+ T cell response to influenza. *Nat. Med.* 21: 1216–1222.
62. Iams, K. P., J. R. Young, V. Nene, J. Desai, P. Webster, O. K. ole-MoiYoi, and A. J. Musoke. 1990. Characterisation of the gene encoding a 104-kilodalton microneme-rhoptry protein of Theileria parva. *Mol. Biochem. Parasitol.* 39: 47–60.
63. Skilton, R. A., R. P. Bishop, C. W. Wells, P. R. Spooner, E. Gobright, C. Nkonge, A. J. Musoke, M. Macklin, and K. P. Iams. 1998. Cloning and characterization of a 150 kDa microsphere antigen of Theileria parva that is immunologically cross-reactive with the polymorphic immunodominant molecule (PIM). *Parasitology* 117: 321–330.
64. Seifried, A., J. Schultz, and A. Gohla. 2013. Human HAD phosphatases: structure, mechanism, and roles in health and disease. *FEBS J.* 280: 549–571.
65. Frasse, P. M., and A. R. Odom John. 2019. Haloacid dehalogenase proteins: Novel mediators of metabolic plasticity in *Plasmodium falciparum*. *Microbiol. Insights* 12: 1178636119848435.
66. Bishop, R., E. Gobright, V. Nene, S. Morzaria, A. Musoke, and B. Sohanpal. 2000. Polymorphic open reading frames encoding secretory proteins are located less than 3 kilobases from Theileria parva telomeres. *Mol. Biochem. Parasitol.* 110: 359–371.
67. Schmuckli-Maurer, J., C. Casanova, S. Schmied, S. Affentranger, I. Parvanova, S. Kang'a, V. Nene, F. Katzer, D. McKeever, J. Müller, et al. 2009. Expression analysis of the *Theileria parva* subtelomere-encoded variable secreted protein gene family. *PLoS One* 4: e4839.
68. Baldwin, C. L., B. M. Goddeeris, and W. I. Morrison. 1988. T-cell clones in immunoparasitology. *Parasitol. Today* 4: 40–45.
69. Pelle, R., S. P. Graham, M. N. Njahira, J. Osaso, R. M. Saya, D. O. Odongo, P. G. Toye, P. R. Spooner, A. J. Musoke, D. M. Mwangi, et al. 2011. Two *Theileria parva* CD8 T cell antigen genes are more variable in buffalo than cattle parasites, but differ in pattern of sequence diversity. *PLoS One* 6: e19015.
70. Connelley, T. K., N. D. MacHugh, R. Pelle, W. Weir, and W. I. Morrison. 2011. Escape from CD8+ T cell response by natural variants of an immunodominant epitope from *Theileria parva* is predominantly due to loss of TCR recognition. *J. Immunol.* 187: 5910–5920.
71. Oura, C. A. L., B. B. Asiimwe, W. Weir, G. W. Lubega, and A. Tait. 2005. Population genetic analysis and sub-structuring of *Theileria parva* in Uganda. *Mol. Biochem. Parasitol.* 140: 229–239.

Key Points

- Multiple CD4 T cell Ags are identified by screening a *Theileria parva* peptide library.
- Ags have diverse properties and include polymorphic and conserved proteins.
- Parasite infection and viral vector–delivered Ag induce similar CD4 responses.