

Approaches to vaccination against *Theileria parva* and *Theileria annulata*

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Summary

Despite having different cell tropism, the pathogenesis and immunobiology of the diseases caused by *Theileria parva* and *Theileria annulata* are remarkably similar. Live vaccines have been available for both parasites for over 40 years, but although they provide strong protection, practical disadvantages have limited their widespread application. Efforts to develop alternative vaccines using defined parasite antigens have focused on the sporozoite and intracellular schizont stages of the parasites. Experimental vaccination studies using viral vectors expressing *T. parva* schizont antigens and *T. parva* and *T. annulata* sporozoite antigens incorporated in adjuvant have, in each case, demonstrated protection against parasite challenge in a proportion of vaccinated animals. Current work is investigating alternative antigen delivery systems in an attempt to improve the levels of protection. The genome architecture and protein-coding capacity of *T. parva* and *T. annulata* are remarkably similar. The major sporozoite surface antigen in both species and most of the schizont antigens are encoded by orthologous genes. The former have been shown to induce species cross-reactive neutralizing antibodies, and comparison of the schizont antigen orthologues has demonstrated that some of them display high levels of sequence conservation. Hence, advances in development of subunit vaccines against one parasite species are likely to be readily applicable to the other.

KEYWORDS

CD8 T cell, neutralizing antibody, *Theileria annulata*, *Theileria parva*, vaccination

1 | INTRODUCTION

Theileria are tickborne apicomplexan parasites found in tropical and subtropical regions of the world, where they predominantly infect ruminants.^{1–3} Wild and domestic ruminants harbour a large number of *Theileria* species, but only a few species, most notably *Theileria parva* and *Theileria annulata* in cattle and *Theileria lestoquardi* (previously also known as *Theileria hirci*) in sheep, are associated with severe clinical disease in farm animals.^{4–8} The diseases caused by these parasites are of major economic importance in the regions where they occur. Their geographical distribution is determined largely by that of the respective tick vector species. *Theileria parva*, which is transmitted

predominantly by *Rhipicephalus appendiculatus* ticks, occurs in eastern and southern Africa, whereas *T. annulata*, transmitted by several species of *Hyalomma* ticks, occurs around the Mediterranean basin, north-east Africa, the Middle East, India and southern Asia. *Theileria lestoquardi*, which is closely related to *T. annulata* and is transmitted by the same tick species,^{9,10} appears to have a more restricted distribution, reports of infection being confined mainly to the Middle East and north-east Africa. *Theileria parva* and *T. annulata* also infect the African buffalo (*Syncerus caffer*) and the Asian buffalo (*Bubalus bubalus*), respectively. The latter sometimes suffer mild clinical disease,¹¹ whereas *T. parva* is nonpathogenic in the African buffalo, but infected buffalo represent an important wildlife reservoir for infection of cattle.¹²

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The pathogenic *Theileria* species cause acute lymphoproliferative diseases, with high levels of morbidity and mortality in susceptible populations of animals.^{6,13,14} Like malaria parasites, *Theileria* undergo sequential development in nucleated cells and erythrocytes, but pathogenicity is largely attributable to parasite development during the nucleated cell stage. *Theileria* invade leucocytes, but unlike most other apicomplexan parasites, they reside free within the cytosol of the host cells.¹⁵ Development to the schizont stage induces activation and proliferation of the infected host leucocytes,¹⁶ and, by associating with the mitotic spindle during cell division, the parasites are able to divide at the same time as the host cells, ensuring that infection is retained in the daughter cells.^{17–19} This process facilitates rapid parasite multiplication prior to differentiation to the erythrocyte-infective merozoite stage. In susceptible animals, large numbers of infected cells are found in the local lymph node draining the site of infection, from which they disseminate throughout the lymphoid system and to nonlymphoid tissues.²⁰ Infection usually results in death within 3–4 weeks. The mode of replication of the schizont stage of *T. parva*, *T. annulata* and *T. lestoquardi* enables the parasitized cells of these species to be cultured in vitro as continuously growing cell lines.²¹ These *Theileria* are frequently referred to as “transforming species”. Other species such as *Theileria mutans* and *Theileria veliferi*, which rarely cause disease, multiply predominantly during the intra-erythrocytic stage of development and undergo limited replication in nucleated cells (reviewed in Ref. 22).

The intra-erythrocyte piroplasm stage of *T. parva* undergoes little or no multiplication, whereas there is some replication of *T. annulata* piroplasms,²³ which is associated with higher levels of infection of erythrocytes. Infections with *T. annulata* may result in moderate anaemia and occasionally jaundice, although pathology produced by the schizont stage is usually the primary cause of mortality by both species.

Because of the acute and fatal nature of the *Theileria* infections in susceptible stock, control of the diseases is particularly challenging. In the past, prevention of tick infestation by application of acaricides has been used successfully to prevent disease. However, the need for almost continuous use of these chemicals has proved to be expensive and difficult to sustain and runs the risk of selecting acaricide-resistant tick populations. A single therapeutic compound (buparvaquone, marketed as Buparvex) is available,²⁴ but its use is limited by cost and the need to treat animals during the early stages of disease to be effective. Moreover, there are recent reports of the emergence of drug-resistant strains of *T. annulata*.^{25,26}

Due to the shortcomings of these control measures, it has long been recognized that vaccination is the most sustainable option for control of these diseases. Live vaccines were produced for *T. parva* and *T. annulata* over 40 years ago,^{5,27,28} but have a number of practical disadvantages that have limited their use in many areas. Efforts to develop vaccines based on use of defined antigens have so far met with limited success. These recent studies have been the subject of several recent reviews.^{29–31} Herein, we will discuss the current status of vaccination against *T. parva* and *T. annulata* and consider the potential value of comparative studies of these parasites for future development of improved vaccines.

2 | THEILERIA PARVA AND THEILERIA ANNULATA INFECT DIFFERENT CELL TYPES BUT CAUSE SIMILAR IMMUNOPATHOLOGY

The ability to infect bovine cells in vitro with tick-derived sporozoites has enabled the cell tropism of the different *Theileria* species to be determined. Early studies demonstrated that *T. parva* sporozoites can bind to and infect B and all subsets of T lymphocytes in vitro with similar efficiency, whereas *T. annulata* was found to infect monocytes and B lymphocytes but not T lymphocytes.^{32,33} Subsequent analyses of the cells infected by *T. parva* in vivo showed that the vast majority of the infected cells in animals undergoing primary infection were T cells, of both CD4 and CD8 T-cell lineage.³⁴ Moreover, experiments in which purified cell populations were infected in vitro with sporozoites and then administered to the autologous animals after 24 hours (when no viable sporozoites remained) demonstrated that infected T cells produced lethal infections, whereas infected B cells resulted in transient mild infection from which they recovered.³⁵ In the animals that received *T. parva*-infected B cells, infection was first detected in the regional lymph node around the same time as in animals receiving infected T cells, but they were able to clear the infection around 11–12 days after infection and were subsequently immune to parasite challenge. We have recently shown that animals inoculated with infected autologous T cells that have been cultured in vitro for 6 weeks or more also develop similar mild self-limiting infections (Morrison WI and Connelley T, unpublished data). Based on these findings, we have concluded that transformation of the infected cells alone is not sufficient for virulence and that additional as yet undefined properties of recently infected T cells enable them to produce disease. Recent studies have identified a Zambian isolate of *T. parva* that infects CD8 T cells but not CD4 or γ/δ T cells.³⁶ Infection with this isolate results in disease, although the authors suggested that it is of lower virulence than other isolates. The relative contribution of infected monocytes and B cells to infections with *T. annulata* in vivo has not been determined.

Despite the different cell tropisms of *T. parva* and *T. annulata*, the pathology they produce is remarkably similar. In both infections, the parasitized cells migrate from the site of infection and become disseminated throughout the lymphoid system. In both cases, there is also evidence of an early powerful nonspecific T-cell response in animals experimentally infected with a lethal dose of sporozoites, but this response appears to be ineffective in controlling the infection. In the case of *T. parva*, the regional lymph node was found to contain 15%–20% lymphoblasts at a time when there was <1% parasitized cells.³⁷ A majority of these cells were CD8 T cells, including a phenotypically unusual subset of CD2⁻CD8⁺ cells; they did not show any cytotoxic activity against parasitized cells and also failed to respond to antigenic or mitogenic stimulation in vitro. Separate studies of animals infected with *T. annulata* revealed a broadly similar picture.^{38–40} These observations have not been investigated further, nor has there been any comparison of the responses to infections induced by needle and tick challenge. Nevertheless, currently available data indicate that

the progressive nature of infections with the two parasite species is not due to a failure to stimulate an immune response but rather that the infection stimulates a dysregulated immune response that fails to differentiate to generate appropriate effector functions. This presumably reflects a strategy by the parasites to delay parasite clearance. What is remarkable is that infection of different cell types by the two *Theileria* species results in a very similar outcome. Further research using contemporary methodologies is required to understand the molecular basis of these immune responses, to better inform what is required for appropriate differentiation of a protective vs a nonprotective immune response.

3 | LIVE VACCINES

3.1 | Vaccination with parasitized cell lines

The advent in the 1960s of cell culture systems to propagate *Theileria*-infected cells as continuously growing cell lines in vitro opened up an obvious opportunity to investigate the use of these cells for vaccination. However, the results obtained with *T. annulata* and *T. parva* were very different. Administration of 10^5 or fewer *T. annulata*-infected cells resulted in immunity to parasite challenge, although prolonged in vitro passage of the cell lines was required to avoid production of clinical disease by the vaccine.^{5,27,41} Initial studies in Israel in the early 1970s resulted in a national vaccine programme for *T. annulata* using cultured cells. Similar vaccines were subsequently developed in several other countries using cell lines derived from local parasite isolates. These vaccines are generally produced in batches that are cryopreserved in aliquots in liquid nitrogen and thawed immediately before use. A dose of approximately $1-5 \times 10^6$ cells (equivalent to 1–5 mL of cultured cells) is used, which allows considerable leeway for cell loss during freezing and thawing of the vaccine.⁵

By contrast to *T. annulata*, similar studies with *T. parva* demonstrated that a dose of 10^8 cultured cells was required to generate immunity in all animals.⁴² Similar to *T. annulata*, in vitro passage of the cells (>50 passages) was required to avoid production of disease by the inoculated cells. As 10^8 cells represented >100 mL of culture, this method of vaccination was not considered economically viable.

Subsequent studies demonstrated that, for both parasite species, transfer of the parasites from the donor culture cells into cells of the recipient animals is required for successful induction of immunity and that this transfer occurs at much lower frequency with *T. parva* than with *T. annulata*.^{43,44} This reflects a requirement of protective T-cell responses to recognize parasite antigens in the context of the major histocompatibility complex (MHC) of the recipient animal. Analyses of immune responses in vaccinated animals demonstrated that animals generated a CD8 T-cell response directed against alloantigens on the inoculated cells at 7–10 days, followed by a second CD8 T-cell response about a week later, which was parasite specific and restricted by the MHC of the recipient animal. The mechanisms by which the schizonts transfer from one cell to another are not known, nor is it clear why this occurs with greater efficiency in *T. annulata*.

3.2 | Vaccination by infection with sporozoites

The unfeasibility of using cultured parasites for vaccination against *T. parva* prompted alternative methods of immunization with live parasites. With the development of methods to cryopreserve large stocks of sporozoites, in the form of homogenized infected ticks,⁴⁵ titrations of this material in cattle were undertaken with the aim of identifying a dose that would reproducibly result in mild transient infections and immunity. However, this was not achievable because the lowest doses that produced infection in all animals still resulted in severe reactions in some of the animals.⁴⁶ An alternative approach involving infection and simultaneous treatment with oxytetracycline was developed, which successfully achieved mild transient infections in all animals.^{28,47} Use of a long-acting formulation of oxytetracycline that provided 5–6 days of activity was required to control the infection. This so-called infection and treatment immunization procedure resulted in long-lasting immunity in all animals against high challenge doses of the *T. parva* isolate used for immunization, but only a proportion of immunized animals withstood challenge with other parasite isolates.^{48,49} However, based on a series of experiments involving immunization and challenge of cattle with different combinations of parasite isolates, a mixture of three isolates was identified (known as the Muguga cocktail), which when used to immunize cattle gave broad protection against experimental challenge with different parasite isolates and against field challenge with *T. parva*. Despite evidence of efficacy,⁵⁰ until recently use of the Muguga cocktail vaccine in the field has been limited.

3.3 | Shortcomings of live *Theileria* vaccines

3.3.1 | Practical constraints

Vaccination using the Muguga cocktail requires production of three large batches of *T. parva* sporozoites by feeding ticks on cattle infected with each parasite isolate, and each batch needs to be carefully titrated in cattle to determine a dose that will reproducibly infect and immunize all animals but will not break through the tetracycline treatment. This complex protocol coupled with the requirement for a liquid nitrogen cold chain to distribute the vaccine presents challenges for quality control and marketing. However, recent initiatives have led to increased field uptake. This has included the establishment of a centre for vaccine production and systems to facilitate distribution of the vaccine.

3.3.2 | Parasite strain-restricted immunity

As referred to above, vaccination against *T. parva* by infection and treatment was found to require incorporation of three parasite isolates in the vaccine to provide immunity against field challenge. This followed on from field studies in which animals immunized with a single parasite isolate were not protected, providing the first convincing evidence of antigenic heterogeneity in *T. parva*.⁵¹ More extensive testing of the Muguga cocktail vaccine has demonstrated that it does

not provide complete protection against field challenge in all circumstances, in particular against challenge with buffalo-derived parasites.^{52,53} Indeed in one study, vaccinated animals introduced into an area grazed only by buffalo showed no protection.⁵² These findings are consistent with sequencing data on parasite genes encoding two polymorphic antigens (Tp1 and Tp2) and genomewide SNP density, which revealed much greater genotypic diversity in parasites isolated from buffalo compared with those of cattle origin.⁵⁴

The results of two recent studies of the Muguga cocktail vaccine, one involving genomic sequencing of the three component parasites⁵⁵ and the other based on high-throughput sequencing of PCR amplicons of six genes encoding *T. parva* antigens (including Tp1 and Tp2),⁵⁶ have indicated that the vaccine contains only a small component of the genetic and antigenic diversity detected in field populations of *T. parva*. Each of the three parasite isolates in the Muguga cocktail exhibited very limited diversity, and two of them (Muguga and Serengeti) showed a remarkably high level of sequence similarity, but differed significantly from the third isolate (Kiambu). The Serengeti parasite was originally isolated from a buffalo and adapted to tick transmission between cattle following several tick passages. However, the results of these two studies both suggested that the Serengeti isolate had at some point become contaminated with parasites from the Muguga isolate. Amplicon sequencing and satellite DNA typing also demonstrated that the vaccine components contained minor genotypic components present at <5% within the vaccine parasites.⁵⁶ If these minor components contribute to the broad protective capacity of the vaccine, then the possibility that these components might not be present in all vaccine batches or indeed all vaccine doses is of concern regarding standardization of vaccine content. The authors proposed formulating an alternative vaccine comprising a mixture of antigenically divergent parasite clones, to standardize the content of the vaccine and potentially enhance its ability to generate broadly cross-reactive immunity. This material would have to be derived through tick passage, and the influence on parasite homogeneity through sexual recombination would need to be investigated.

It is of note that antigenic heterogeneity among isolates of *T. annulata* has also been documented in a number of early studies, which showed incomplete cross-protection between some isolates, with a proportion of the animals succumbing to disease (reviewed in Ref. 4,5). Moreover, initial cell line vaccine testing in Israel showed that vaccinated animals challenged with heterologous parasite isolates developed more severe clinical reactions than those receiving homologous challenge, although the animals survived. More recently, experiments involving immunization of cattle with cell lines infected with Tunisian *T. annulata* isolates have indicated that the passage history of the cell line can influence the level of protection against heterologous isolates⁵⁷; cattle immunized with cell lines that had undergone prolonged passage suffered more severe reactions following challenge than those immunized with the same cell lines at an earlier level of passage. Analyses of these cell lines with isoenzymes and polymorphic genotypic markers indicated that prolonged passage resulted in reduced genetic diversity in the parasites within the cell lines, and these authors suggested that this loss in diversity may account for the reduced

capacity to provide protection against challenge with heterologous parasite isolates. Nevertheless, the reasons why strain-restricted immunity, as observed with *T. parva*, is not a major issue when vaccinating cattle against *T. annulata* are unclear.

3.3.3 | Acceptability of live vaccines

A key feature of infections with *Theileria* parasites is their ability to establish persistent infections in the face of immune responses that control the infection. In the case of *T. parva* and *T. annulata*, persistent infections (referred to as the carrier state) are usually not detectable microscopically but can be revealed by polymerase chain reaction (PCR) assays and can be transmitted by ticks.⁵⁸ The live parasites used for vaccination often retain the ability to establish a carrier state, although some of the *T. annulata* cell line vaccines that have been subjected to prolonged *in vitro* passage are claimed to have lost this property. Nevertheless, there has been great reluctance to use live parasites originating in one country to vaccinate animals in other countries, because of perceived risks of introducing “foreign parasite strains” of different antigenic composition or virulence. Hence, for *T. annulata*, each country has tended to develop its own cell line vaccine from a local parasite isolate. In the case of *T. parva*, use of the Muguga cocktail vaccine has been slow to gain acceptance outside Kenya and Tanzania. In the longer term, this issue could potentially be addressed by genetically modifying the parasite such that the carrier status is no longer maintained.

4 | APPROACHES TO DEVELOPMENT OF SUBUNIT VACCINES

Because of the limitations of available live vaccines, efforts have been made to develop alternative vaccines based on the use of defined antigens. This has necessitated studies to understand the immune responses to the parasites and their role in immune protection. Much of this work has focused on the sporozoite and schizont developmental stages.

4.1 | Protective immune responses to schizont-infected cells

4.1.1 | Immune protection

There is a large body of evidence indicating that immunity generated by infection with *T. parva* or *T. annulata* is mediated by cellular immune responses directed against schizont-infected leucocytes. This information has been reviewed elsewhere^{29,30} and will therefore only be summarized briefly here.

Because animals can be immunized by administration of schizont-infected cell lines, immunity is clearly not dependent on exposure to preschizont stages of the parasite. Moreover, immunization with live parasites, either cell lines or sporozoites, does not prevent establishment of infection, but rather schizont-infected cells are often detected

transiently 8–10 days after parasite challenge of immunized animals, indicating that immunity operates against this stage of the parasite.

Analyses of the immune responses *ex vivo* following challenge of *T. parva*-immunized animals have demonstrated MHC-restricted cytotoxic CD8 T-cell responses against autologous *T. parva*-infected cells, coinciding with parasite clearance.^{59,60} Two further observations provided evidence that these T cells are key mediators of immunity to *T. parva*. First, transfer of responding CD8 T cells (but not CD4 T cells), from immune to naïve identical twin calves, was found to confer protection against parasite challenge in the naïve recipients.⁶¹ Second, the CD8 T-cell responses showed parasite strain specificity that varied between animals immunized with the same parasite isolate, and correlated closely with the observed immunity following challenge with a cloned heterologous parasite strain.^{62,63} Further studies have demonstrated high frequencies of parasite-specific CD8 T-cell precursors in the memory T-cell populations of immune animals,⁶³ and the specificities of these T cells have been studied in detail using CD8 T-cell lines and clones derived from the memory populations.^{64,65} Analyses of T-cell lines obtained from immune animals by stimulation with autologous parasitized cells have also identified strong parasite-specific CD4 T-cell responses, which recognize antigen presented on the surface of infected leucocytes.^{66,67} Findings from *in vitro* experiments involving mixing of CD4 and CD8 T cells from immune or naïve animals have indicated that the parasite-specific CD4 T cells may provide help for induction and recall of parasite-specific CD8 T-cell responses.⁶⁸

While the role of T-cell responses in immunity to *T. annulata* has not been examined in the same detail, available evidence indicates

a very similar profile of responses, namely cytotoxic CD8 T-cell responses detected *ex vivo* following immunization and challenge with the parasite and the presence of CD4 and CD8 T cells specific for autologous parasitized cells in T-cell lines generated *in vitro* from immune animals.^{69,70}

4.1.2 | The antigens recognized on schizont-infected cells

A series of studies using *T. parva*-specific CD8 T-cell lines to screen for recognition of cells transfected with parasite cDNAs has identified 10 antigens (Table 1) that are recognized by CD8 T cells from immune cattle.^{56,71} The genes encoding these antigens are unrelated and are distributed across the genome. Some are predicted to be orthologues of other parasite and/or mammalian genes, whereas others have no detectable orthologues. A striking feature of the antigen screening results was that animals of different MHC genotypes tended to detect different *T. parva* antigens, and, within the animals studied, only one of the antigens (Tp2) was recognized by T cells from animals of several different MHC genotypes. Epitope screening, in most cases, also identified a single dominant epitope recognized by T cells restricted by a given MHC allele.⁷² However, animals of some MHC types do not recognize any of these antigens. The results of these studies collectively indicate that a large number of *T. parva* proteins are capable of eliciting CD8 T-cell responses and that many of these target antigens have not yet been identified.

Detailed analyses of the responses to two of the antigens, Tp1 and Tp2, which are recognized on the A18 and A10 MHC backgrounds,

TABLE 1 Level of amino acid sequence conservation between orthologues of schizont vaccine antigens in *Theileria parva* and *Theileria annulata*

<i>Theileria parva</i>			<i>Theileria annulata</i>			
Antigen	Amino acids	Epitope sequence	Antigen ^a	Amino acids	Epitope sequence ^b	Ta/Tp amino acid identity ^c
Tp1	543	²¹⁴ VGYPKVKEEML ₂₂₄	Ta1	529	²¹⁴ YKYPNIKQEML ₂₂₄	52%
Tp2	175	²⁷ SHEELKLGML ₃₇ ⁴⁰ DGFDRDALF ₄₈ ⁴⁹ KSSHGMGKVGK ₅₉ ⁹⁶ FAQSLVCVL ₁₀₄ ⁹⁸ QSLVCVLMK ₁₀₆ ¹³⁸ KTSIPNPCKW ₁₄₇	Ta2	178	²⁸ KDEELDAMGML ₃₈ ⁴¹ DLNKELLFQ ₄₉ ⁵⁰ QTSHILTKVGK ₆₀ ⁹⁷ FAASIHCV ₁₀₅ ⁹⁹ ASIHCVANK ₁₀₇ ¹³⁹ KESIPNPCKDW ₁₄₈	60%
Tp3	265	Unknown	Ta3	265		77%
Tp4	579	³²⁸ TGASIQTTL ₃₃₆	Ta4	520	³²⁸ TGASIQTTL ₃₃₆	96%
Tp5	155	⁸⁷ SKADVI ₉₅	Ta5	155	⁸⁷ SKADVI ₉₅	98.1%
Tp6	277	Unknown	Ta6	277		98.5%
Tp7	721	²⁰⁶ EFISFPISL ₂₁₄	Ta7	722	²⁰⁶ EFISFPISL ₂₁₄	97.5%
Tp8	434	³⁷³ CGAELNHFL ₃₈₁	Ta8	409	³⁵² CGAELNHYL ₃₆₀	89%
Tp9	335	⁶⁷ AKFPGMKKS ₇₅	Ta9	336	⁶⁴ SKFPMRMG ₇₂	64%
Tp10	443	⁴¹⁹ NNPELIPVL ₄₂₇	Ta10	447	⁴²³ NNPELIPVL ₄₃₁	92%

^aOnly Ta5 and Ta9 (bold) confirmed as CD8 T-cell antigens in *T. annulata*.

^bDivergent amino acid residues are highlighted in grey.

^cThe percentage of amino acid residues conserved between the *T. parva* and *T. annulata* orthologues, based on the reference genome sequences.

respectively, demonstrated that in each case, approximately 70% of the parasite-specific CD8 T cells in MHC-homozygous immune animals were specific for the respective antigen and that these animals did not recognize the other defined antigens.⁶⁵ Subsequent work demonstrated that these antigens are highly polymorphic and that the respective T-cell responses are often parasite strain-restricted. Elsewhere, we have discussed in further detail how this observed focusing of the CD8 T-cell response on highly dominant polymorphic T-cell epitopes is a key determinant of parasite strain specificity of CD8 T-cell response.^{65,73} However, recent unpublished studies (H. Hemmink, W.I. Morrison, P. Toye, T. Sitt and W. Weir, manuscript in preparation) have shown that several of the other *T. parva* CD8 T-cell antigens are highly conserved among field isolates of the parasite.

Similar large-scale antigen screening has not been undertaken with T cells specific for the other pathogenic *Theileria* species. However, screening for recognition of the orthologues of the 10 *T. parva* antigens in *T. annulata*⁷⁴ and *T. lestoquardi*⁷⁵ has been conducted with a limited number of CD8 T-cell lines from cattle and sheep, respectively. In each case, positive results were obtained with two antigens (Tp5 and Tp9 orthologues in *T. annulata* and Tp8 and Tp9 orthologues in *T. lestoquardi*). An additional *T. annulata* antigen was identified by screening a small number of parasite cDNAs. Analyses of sequences of Tp9 and the orthologue in *T. annulata* in different parasite isolates have shown that this antigen is highly polymorphic in both parasite species^{56,74} and that the corresponding CD8 T-cell lines exhibit parasite strain specificity. Although the repertoire of antigens recognized by CD8 T cells specific for these parasites is far from complete, these initial findings suggest that there is likely to be substantial commonality in the gene products that they recognize. Hence, information from one species can help to focus efforts on antigen screening in the other species.

4.1.3 | Vaccination with schizont antigens

Five of the *T. parva* antigens recognized by CD8 T cells, including Tp1 and Tp2, have also been tested for their ability to induce immune responses and protection against parasite challenge.⁷¹ These experiments involved the use of prime-boost protocols involving priming with plasmid DNA or recombinant canarypox viruses followed by a single boost with replication-defective recombinant vaccinia viruses (Ankara strain). Animals were immunized simultaneously with five of the antigens in separate DNA or viral constructs. Most of the immunized animals (19 of 24) exhibited readily detectable antigen-specific CD8⁺ T-cell IFN- γ responses following immunization, but the CD8 T cells only exhibited detectable cytotoxic activity for parasitized cells in four of the 24 immunized animals.⁷¹

The animals were challenged along with unimmunized controls with a lethal dose of sporozoites 3 weeks after the booster immunization. Nine of the 19 immunized animals that generated a specific CD8 T-cell IFN- γ response, including the four whose CD8 T cells exhibited cytotoxicity, survived the challenge, although some of them developed moderate-to-severe clinical reactions prior to recovery. There was a significant association between survival and the induction of a cytotoxic CD8 T-cell response. Thus, although the immunization

protocols used in this study clearly did not induce a level of immunity that is of practical value for vaccination, they resulted in reduction in severity of disease and survival in a proportion of the animals.

Subsequent experiments in which similarly immunized cattle were treated with DNA-encoded FLT3L and GM-CSF, in an attempt to increase targeting of the expressed antigen to dendritic cells, failed to enhance protection.⁷⁶

The results obtained from these initial vaccination experiments indicated that the vaccine-induced CD8 T-cell responses to schizont antigens may not be fully functionally competent to provide protection against parasite challenge. Current studies are investigating differences in the function of the specific CD8 T cells induced by live parasites and the subunit proteins and exploring the utility of alternative antigen delivery systems, including improved replication-defective poxvirus vectors and other alternative viral vectors. The potential contribution of other cell subsets, such as CD4 T cells, to protection is also being investigated.

4.2 | Protective immune responses to sporozoites

Although infections with *T. parva*⁷⁷ or *T. annulata*⁷⁸ induce only low levels of antibody against sporozoite antigens, antibodies capable of fully neutralizing the infectivity of sporozoites in vitro have been detected in animals subjected to repeated sporozoite challenge. Moreover, monoclonal antibodies with neutralizing activity (nmAbs) have been produced for both parasites by immunizing mice with sporozoites.^{79–81} The majority of such antibodies recognize a sporozoite surface protein, called p67 in *T. parva*⁸² and SPAG1 in *T. annulata*.⁸¹ Allelic variants of the SPAG1 protein are detected in parasites derived from both cattle and Asian buffalo.⁸³ While allelic variants of p67 have been detected, they appear to be found primarily in parasites derived from the African buffalo rather than from cattle-derived parasites.^{84,85}

The p67⁸⁶ and SPAG1⁸¹ proteins are encoded by single copy genes and consist of ~700 and ~900 amino acid residues, respectively. They are major components of the sporozoite surface membrane. Like the circumsporozoite protein (CSP) of *Plasmodium* sporozoites,⁸⁷ the p67/SPAG1 proteins play a role in host cell recognition and entry, but they are not expressed in the schizont stage. Recombinant p67⁸⁸ and SPAG1^{89,90} incorporated in adjuvant induce sporozoite neutralizing activity in cattle and induce immunity to sporozoite needle challenge in a proportion of immunized cattle. In most of these experiments, the sporozoite challenge dose was titrated to provide less than an LD₁₀₀.

A large number of cattle laboratory challenge experiments have been carried out with various different forms of recombinant p67 with different adjuvants and vectored antigen delivery systems, which has been recently reviewed.³¹ In brief, recombinant protein gave superior results to the vectored systems used and immunity to severe ECF ranged from 20% to ~70%. A range of different clinical responses to challenge was observed, from no-reaction to mild, moderate and severe disease. It is likely that in the first of these categories of animals, no infection occurred, as these cattle were negative by PCR. Immunity induced by SPAG1 and different forms of SPAG1 has been less well

studied, but as with p67 has been found to induce significant levels of immunity (~50%) to challenge.⁹⁰ Interestingly, the SPAG1 protein has been shown to synergize with the protective efficacy of the Tams-1 protein^{91,92} and a live attenuated *T. annulata* cell line,⁹³ indicating that there is merit in assessing the role of multiple parasite antigens in increasing the efficacy of candidate vaccine antigens.

Expression of recombinant p67 in a native and stable form remains a technical challenge. Mapping of linear B-cell epitopes on p67 has revealed that the bovine immune response to recombinant full-length p67 is primarily directed to N- and C-terminal domains, which also harbour linear epitopes recognized by nmAbs.⁹⁴ This has led to the demonstration that an easy-to-produce 80 amino acid peptide at the C-terminal end of p67, called p67C, induces the same level of immunity to full-length p67.⁹⁵ This immunogen is now being used to further optimize protective immune responses to ECF. A 108 amino acid C-terminal peptide of SPAG1 fused to hepatitis B core antigen has also been shown to induce immunity equivalent to that of full-length SPAG1.⁸⁹

The polymorphic immunodominant molecule (PIM) in *T. parva* is also a target of murine nmAbs,^{96,97} but as cattle do not make neutralizing antibodies to full-length recombinant PIM, this antigen has not been tested in challenge experiments.^{98,99} PIM is expressed in both sporozoites and schizonts. An orthologue of this protein, called TaSP¹⁰⁰ and TISP,¹⁰¹ is present in *T. annulata* and *T. lestoquardi*. The PIM antigen is rich in glutamine and proline amino acid residues and consists of a complex central domain that is variable in length flanked by conserved N- and C-terminal sequences.⁹⁸ Sequence variation in the central domain results in the PIM antigen ranging in size from 62 to 112 kDa. In contrast, the TaSP antigen does not exhibit the extreme size polymorphism of PIM and is about 36 kDa in size.¹⁰⁰ Although allelic variants are found, the TaSP variants do not contain the complex central domain observed in the PIM antigen. As its name implies, cattle mount a strong antibody response to PIM and this antigen is used as a serological diagnostic test as a marker of *T. parva* infection.¹⁰² The TaSP protein has also been used to develop a serological diagnostic test as a marker of *T. annulata* infection.¹⁰³

5 | COMPARATIVE THEILERIA PARVA AND THEILERIA ANNULATA GENOMICS

Available information on the antigens recognized by protective immune responses in the two *Theileria* species indicates that the immune responses are directed to the products of orthologous genes and in some instances have provided evidence of antigenic cross-reactivity. Herein, we will explore this further to consider whether there is scope for inducing protective responses that are active against both parasite species.

5.1 | Conservation in genome architecture and gene content

The genome architecture and protein-coding capacity of *T. parva* and *T. annulata* are remarkably similar. *Theileria parva*¹⁰⁴ and *T. annulata*¹⁰⁵

each encode four Mbp-sized nuclear chromosomes, a small linear mitochondrial (~7 kbp)^{106,107} and circular apicoplast (~39 kbp) genome with a total genome size of ~8.35 Mbp. Gene density is very high in both organisms, with *T. parva* predicted to code for a larger number of chromosomal encoded proteins (4035) than *T. annulata* (3792). It should be highlighted that these data were generated over a decade ago and there has been much improvement in bioinformatics and sequencing tools in the interim. The reference *T. parva* genome has been resequenced and re-annotated and gene models were refined using RNAseq data from the schizont stage (cited in¹⁰⁸), which has resulted in substantial change in exon-intron boundaries and in the discovery of additional protein-coding genes. A similar exercise is being undertaken for *T. annulata* (A. Pain, personal communication). Comparative data on the physical map of chromosomes suggest that the total size of the *T. parva* genome can vary between different parasite isolates.¹⁰⁹ The impact of genome size variations on parasite gene content and biology, however, remains to be fully documented. Such data are beginning to accumulate for *T. parva* as more strains are sequenced^{55,110,111} and their impact on genotypic diversity has been briefly reviewed elsewhere.³¹

Both genomes exhibit a highly compact structure. In brief, chromosomal DNA does not contain highly repetitive DNA, and telomeres are short, and noncoding subtelomeric sequences in *T. parva* are simpler in sequence than those found in *T. annulata*.¹⁰⁵ A high proportion of protein-coding genes encode introns that tend to be short, and intergenic regions are also short. The *T. parva* and *T. annulata* genomes exhibit near complete synteny across all four chromosomes, with interruptions due to insertions or deletions of members of the multigene families.^{105,112} The most notable inversion point relates to the *T. parva* Tpr locus, which occupies a region of ~150 kbp within chromosome 3 and consists of a large tandem array of open reading frames. Members of the equivalent gene family in *T. annulata*, Tar, are dispersed across the nuclear genome. There are no interchromosomal rearrangements.

5.2 | Conservation of candidate *Theileria parva* and *Theileria annulata* vaccine antigens

5.2.1 | Sporozoite antigens

Although the p67 and SPAG1 proteins exhibit only 47% sequence identity, there is sufficient conservation of epitopes between them so that anti-p67 serum recognizes SPAG1 in immunoblots and neutralizes in vitro the infectivity of *T. annulata* sporozoites, and vice versa.¹¹³ This functionality even extends to some nmAbs. mAb 23F raised to p67 inhibits *T. annulata* sporozoite infectivity, and mAb 1A7 raised to SPAG1 inhibits *T. parva* sporozoite infectivity. By Pepscan analysis on p67, 1A7 has been shown to bind to the core peptide sequence PSLVITD. The sequence PSLVI is present in SPAG1.¹¹³ mAb 23F binds a conformational epitope in p67, which remains to be mapped. An orthologue of p67/SPAG1 is also present in *T. lestoquardi*, called SLAG1.¹¹⁴ Encoding 723 amino acid residues, the protein shares 42% and 58% sequence identity with p67 and SPAG1, respectively. The higher level of sequence identity with SPAG1 is perhaps not unexpected as

T. lestoquardi is a closer relative to *T. annulata* than *T. parva* (reviewed in Ref. 115) Abs to a fragment of SLAG1 bind to p67 and SPAG1 and SLAG1 also contains the sequence PSLVI, which predicts that 1A7 should bind and inhibit *T. lestoquardi* sporozoites and that these three molecules are antigenically related.¹¹⁵ The ability of p67 and SPAG1 to induce cross-species immunity has been confirmed,¹¹⁶ namely that p67 immunized cattle can exhibit immunity to *T. annulata* sporozoite challenge and *vice versa*. Both antigens induced approximately 50% immunity to the homologous and heterologous sporozoite challenge. The role of SLAG1 as a candidate vaccine antigen remains to be tested, but given that p67 also protects a proportion of cattle against ECF under field conditions, that is, through parasite tick challenge,¹¹⁷ this group of related proteins are prime candidate vaccine antigens.

5.2.2 | Schizont antigens

As discussed above, CD8 T-cell responses specific for *T. annulata* have been shown to recognize orthologues of two of the *T. parva* CD8 T-cell target antigens identified to date. Moreover, the amino acid sequence of the epitope identified in one of these antigens (Ta5)⁷⁴ was identical to that in the Tp5 protein,⁷² although it was presented by a different, but related MHC class I allele (1*00902 presented Tp5 and 1*02301 presented Ta5). Comparison of the predicted amino acid sequences of the 10 identified *T. parva* CD8 antigens with their *T. annulata* orthologues, based on the respective reference genome sequences, reveals a high level of sequence conservation between the orthologues of a number of the antigens (Table 1). Thus, Tp4, Tp5, Tp6 and Tp7 exhibit >96% sequence identity and Tp8 and Tp10 between 89% and 92% identity with their *T. annulata* counterparts. Remarkably, the sequences of the CD8 T-cell epitopes identified in four of these antigens are also completely conserved between the species. The lower levels of identity observed for Tp1, Tp2 and Tp9 are consistent with previous evidence that these antigens are highly polymorphic in *T. parva* and, in the case of Tp9, also *T. annulata*.

The question of whether or not there is any cross-protection between these *Theileria* species has not been addressed in any recent studies although a review by Neitz in 1957⁴ reported that animals recovered from infection with *T. annulata* remained susceptible to *T. parva*. However, in *T. parva*, CD8 T-cell responses are often dominated by T cells specific for more polymorphic antigens³⁰; hence, any cross-immunity might only be partial and/or only apparent in a subset of animals. Evolutionary divergence of pathogen species within the same host is usually associated with antigenic divergence to minimize interspecies competition. However, as the geographical distributions of *T. parva* and *T. annulata* do not overlap, due to the different distribution of their tick vectors, there may have been minimal selective pressure to maintain antigenic divergence between these species. In the absence of a proven antigen delivery system for induction of protective CD8 T-cell responses with defined antigens, it is currently not possible to determine the protective potential of these conserved antigens. In the meantime, further studies to examine potential cross-protection between the two species, linked to analyses of the specificity of the T-cell responses, may help to shed light on this question.

6 | CONCLUSIONS

As described above, there is a great deal of similarity in the immunopathology, genomics and biology of *T. parva* and *T. annulata*. Similar protective immune responses are directed against the sporozoite and schizont stages of the parasites, and it is remarkable that many candidate sporozoite and schizont antigens are also so similar to each other. Hence, advances in development of subunit vaccines against one parasite species are likely to be readily applicable to the other. The data on antigenic conservation between the two species are contrary to expectation, as early research reported that cattle immunized with live parasites exhibited species-specific immunity⁴, suggesting that unique antigens were likely to play a role in immunity to infection. However, what we now know about the nature of the immune response to *T. parva* and *T. annulata* may offer an explanation for the apparent lack of cross-protection. First, the sporozoite antigens are not highly immunogenic molecules per se. Although there is clear evidence that immunity can be induced experimentally with p67/SPAG1, a single infection event induces very little antibody specific for p67/SPAG1 and it is not clear whether such responses play a role in immunity in animals residing in endemic areas. Hence, this immunity could be described as “unnatural”. Second, there is marked skewing and dominance in the antigenic specificity of CD8 T-cell responses of individual animals, which is influenced by the MHC genotype of the animal, such that strain-specific immunity may be engendered by T-cell responses to one or two dominant epitopes in polymorphic antigens. Therefore, there is a need to re-examine the cross-protective potential of *T. parva* and *T. annulata* in cattle of MHC types known to respond to conserved epitopes. Great strides have been made in unravelling candidate *Theileria* vaccine antigens, and clear progress has been made in developing species-specific vaccines. The shared antigens raise the intriguing possibility of developing multivalent vaccines effective against the major pathogenic bovine and ovine species of *Theileria*.

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