Progress towards understanding the immunobiology of *Theileria* parasites

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(*Received 13 May 2009; revised 29 June 2009; accepted 30 June 2009; first published online 20 August 2009*)

SUMMARY

The pathogenic Theileria species *Theileria parva* and *T. annulata* infect bovine leukocytes and erythrocytes causing acute, often fatal lymphoproliferative diseases in cattle. The parasites are of interest not only because of their economic importance as pathogens, but also because of their unique ability to transform the leukocytes they infect. The latter property allows parasitized leukocytes to be cultured as continuously growing cell lines *in vitro*, thus providing an amenable *in vitro* system to study the parasite/host cell relationship and parasite-specific cellular immune responses. This paper summarizes important advances in knowledge of the immunobiology of these parasites over the last 40 years, focusing particularly on areas of relevance to vaccination.

Key words: Theileria, cell biology, CD8 T cell, antigenic diversity, immunodominance, vaccination.

INTRODUCTION

At the time I was embarking on a career in research in the early 1970s, the recent elucidation of the genetic code and establishment of the basic concepts of humoral and cellular immunity had created great optimism concerning the potential of research to yield novel methods for the control of infectious diseases. In the field of parasitology, this coincided with an influx of basic immunologists and biochemists, attracted by the challenges of unravelling the intricacies of host-parasite relationships and host immune responses. With growing knowledge of the immune system, it was anticipated that an understanding of protective immune responses would allow the identification of antigens that could rapidly be translated into development of new vaccines. However, despite further technological advances in the intervening period, notably in recombinant DNA technology, synthetic protein chemistry and genome sequencing, the number of effective parasite vaccines remains disappointingly small. In hindsight, it is now clear that both the complexities of host protective immune responses and the difficulties of reproducing such responses by vaccination were seriously underestimated. Nevertheless, advances in knowledge of parasite biology and immunology in the intervening period have indicated that the development of novel vaccines remains a tenable objective.

When I first became involved in parasitology research, approximately 30 years ago, Theileria parasites appeared to be excellent candidates for the development of new vaccines. It had long been recognized, both for *Theileria parva* and *T. annulata*, that animals recovering from infection acquired strong immunity to subsequent parasite challenge, albeit with a degree of parasite strain restriction. Moreover, methods of immunization based on the use of live parasites had recently been developed and shown to be effective in the field, but because of practical shortcomings were not seen as long-term solutions. These vaccines had been developed, despite having little or no knowledge of the nature of the immune responses involved in protection or of the biological properties of the parasites responsible for causing disease. Herein, I will attempt to highlight important advances in the knowledge of the immunobiology of these pathogenic Theileria species, over the last 40 years, focusing particularly on areas of relevance to vaccination.

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A BRIEF HISTORICAL PERSPECTIVE

Theileria parasites were first discovered in South Africa about 100 years ago, just prior to publication of the first issue of *Parasitology*. The discovery was made in the course of investigations into a major outbreak of highly fatal disease in cattle, which appeared first in Rhodesia (now Zimbabwe) in 1901 and shortly thereafter in South Africa. Since the region had only recently re-stocked following decimation of the cattle population by the rinderpest pandemic in the mid-1890s, this new disease created widespread alarm among the local communities prompting requests through the Colonial Office for international assistance in indentifying the causal agent (described by Cranefield, 1991). Robert Kock was one of several eminent experts consulted. Based on investigations carried out during a visit to

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doi:10.1017/S0031182009990916 Printed in the United Kingdom

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

Rhodesia in 1902, he erroneously concluded that the agent was a highly virulent form of babesia. Meanwhile, two employees of the then Transvaal government, Arnold Theiler and Charles Lounsbury, were carrying out their own investigations. Through a series of carefully designed experiments with cattle and ticks, by 1903 they had demonstrated that the disease was caused by a new parasite, which was distinct from babesia, and had identified the tick vector as *Rhipicephalus appendiculatus* (Theiler, 1903). This was a remarkable achievement given the tools that were available for such studies at the time. Their findings allowed implementation of control measures based largely on acaricide application and regulation of animal movement. Subsequent studies revealed that the parasite, eventually named *Theileria parva*, was endemic in a large part of East Africa and that it had been introduced into southern Africa by cattle imported from Tanzania as part of the restocking programme following the rinderpest pandemic. The disease in cattle was named east coast fever (ECF). The African buffalo (*Syncerus caffer*) was also shown to be a reservoir of a very similar parasite, initially named *Theileria lawrencei* and now referred to as buffalo *T. parva*. Infected buffalo did not suffer from disease but developed low levels of persistent infection, which could be transmitted to cattle by *R. appendiculatus* ticks causing severe disease similar to ECF (Neitz *et al.* 1955; Young *et al.* 1977). However, in many cases these buffalo-derived parasites do not develop to the erythrocytic stage in infected cattle and are not transmissible by ticks (Schreuder *et al.* 1977).

Around the same time that Theiler and Lounsbury discovered *T. parva*, investigation of a similar disease in southern Russia resulted in the first description in 1904 of another pathogenic species of Theileria in cattle, subsequently named *T. annulata* (Dschunkowsky and Luhs, 1904). Further studies demonstrated that this parasite was present in a large subtropical region extending from the Mediterranean basin through the Middle East to India and West China and that it was transmitted by several species of *Hyalomma* ticks. Subsequent work conducted on both of these parasites over the next 60 years resulted in a more complete understanding of the epidemiology of the diseases and yielded important observations on the ability to immunize cattle with live parasites. However, more detailed knowledge of the immunobiology of the parasites was only possible with the development of *in vitro* culture systems and the advent of molecular technologies.

STUDIES OF CULTURED PARASITIZED CELLS REVEAL A NOVEL MODE OF PARASITE REPLICATION

The development, in the 1960s and 1970s, of methods for *in vitro* cultivation of leukocytes infected with *T. parva* and *T. annulata* (Hulliger, 1965; Brown *et al.* 1973) was a key advance that has underpinned much of the subsequent research on the cell biology and immunology of these parasites. Cultures of parasitized cells could be established either from tissues of infected cattle or by infection of leukocytes *in vitro* with tick-derived sporozoites and the resultant cell lines could be maintained indefinitely in culture. Early observations by Hulliger revealed that this was possible because of the unique way in which the parasite utilizes the host cell to multiply (Hulliger *et al.* 1964). They showed that the intracellular schizont stage is able to divide at the same time as the host cell, ensuring that infection is retained in the daughter cells. In subsequent ultrastructural studies of host cell invasion by *T. parva* sporozoites, Fawcett and colleagues demonstrated that the parasite escapes from the endocytic vacuole shortly after invasion and resides free within the cytoplasm of the host cell (Fawcett *et al.* 1982, 1984). This enables the parasites to regulate host cell function by secretion of biologically active proteins directly into the cytoplasm.

Fawcett also showed that the parasite associates with the microtubules of the mitotic spindle during cell division (Fawcett *et al.* 1984). These and other observations have demonstrated that clonal expansion of the cells initially infected by the parasites is the principal means of parasite multiplication in the bovine host. Indeed the intra-leukocyte stage of the parasite has a limited capacity to infect other leukocytes, particularly in the case of *T. parva*, where administration of 10^8 allogeneic parasitized cells is required to obtain reproducible infection in the cells of recipient animals (Pirie *et al.* 1970; Emery *et al.* 1982). The ability to achieve infection with smaller numbers (a few thousand) of *T. annulata*-infected leukocytes is the principal reason why vaccination with parasitized cell lines has been feasible for *T. annulata* but not for *T. parva* (Pipano, 1974). However, the mechanism by which infection transfers from donor to recipient cells remains unknown.

THE MOLECULAR BASIS OF HOST CELL TRANSFORMATION

The development of surface markers for subpopulations of bovine leukocytes in the early 1980s allowed identification of the cell types infected by *T. parva* and *T. annulata*. Despite the similarity in the biology of the two parasites and the diseases they produce, their cell tropism was shown to differ. Analysis of the susceptibility of purified subsets of cells to infection *in vitro* showed that *T. parva* infects B and T lymphocytes (including $CD4^+$, $CD8^+$ and TCR γ/δ^+ populations) with similar frequency, whereas *T. annulata* infects mononuclear phagocytes and B lymphocytes (Baldwin *et al.* 1988; Spooner *et al.* 1989). Host cell transformation was shown to

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

be dependent on the continued presence of the parasite, since treatment of infected cells with theilericidal compounds resulted in cessation of proliferation and rapid cell death.

Although the precise mechanisms by which the parasites achieve transformation are still not fully understood, studies of the cell biology of *T. parva*-transformed cell lines have provided insight into the activation status of various signal transduction pathways and their role in maintaining proliferation and viability of the infected cells. Constitutive activation of NFκB appears to be centrally involved in these processes, since treatment of parasitized cells with NFκB inhibitors results in rapid cell death (Palmer *et al*. 1997). Analysis of different steps in the NFκB activation pathway indicated that the parasite directly activates the IκB kinase (IKK) complex leading to phosphorylation of IκB, which is degraded resulting in translocation of NFκB to the nucleus. Particulate accumulations of IKK complex proteins, termed signalosomes have been shown to associate with the surface of the schizont (Heussler *et al*. 2002). The transcriptional activity of NFκB results in host cell activation and upregulation of a number of molecules that render the cells more resistant to apoptosis. There is also evidence that constitutive activation of c-Jun N-terminal kinase (JNK), resulting in phosphorylation of c-Jun and activation of AP-1, contributes to protection against apoptosis and also modulates the endocytic activity of the cells (Lizundia *et al*. 2006). Theileria-transformed cells produce a number of cytokines and there is evidence that both TNFα and GM-CSF can act extrinsically in an autocrine loop to further augment activation of these cell signalling pathways (Guergnon *et al*. 2003; Dessauge *et al*. 2005). Further details on this topic can be found in several recent reviews (Dobbelaere and Kuenzi, 2004; Dessauge *et al*. 2005; Heussler *et al*. 2006).

Additional studies of parasitized cell lines have demonstrated that host cell activation is intimately linked to the differentiation status of the parasite. Shiels and colleagues showed that a brief period of incubation of *T. annulata*-infected cell lines at 41 °C resulted in differentiation of schizonts to undergo merogony and a marked reduction in host cell proliferation (Shiels, 1999). Using this system, they identified two related parasite gene families, the *TashAT* and *SuAT* genes, which were rapidly down-regulated and up-regulated respectively upon induction of parasite differentiation (Swan *et al*. 2001 *a*, *b*). Both families encode proteins with predicted signal peptides and DNA-binding motifs and studies using antibodies raised against selected members demonstrated that they localized to the host cell nucleus (Swan *et al*. 2003; Shiels *et al*. 2004). Transfection of a bovine macrophage cell line with one of the SuAT genes was also shown to result in altered gene expression and cell morphology.

Phenotypic analyses of Theileria-infected cell lines have shown that they retain some lineage-specific markers, although B cells tend to lose expression of surface immunoglobulin (Baldwin *et al*. 1988; Sager *et al*. 1998 *a*). Expression of many other surface proteins and cytokines varies between different cell lines infected with the same parasite. However, certain molecules appear to be consistently up-regulated or down-regulated. For example, IL-10 is expressed by all *T. parva*-infected cells (McKeever *et al*. 1997) and CD14 and CD11b are consistently down-regulated by *T. annulata*-infected cells (Sager *et al*. 1997). The latter were also shown to be refractory to induction of IFN-α, but retained the ability to produce IFN-β (Sager *et al*. 1998 *b*). Since these molecules are all involved as mediators or inhibitors of pro-inflammatory responses, these changes almost certainly reflect deliberate strategies by the parasite to modulate host responses and/or the impact of such responses on the parasitized cells. Hence, our current knowledge of the biology of Theileria-transformed cells, although clearly incomplete, indicates that transformation is likely to be orchestrated by multiple parasite gene products targeting different host functional activities.

HOST CELL TRANSFORMATION IS NOT THE SOLE DETERMINANT OF PARASITE VIRULENCE

Despite the ability of *T. parva* to infect and transform different subsets of lymphocytes *in vitro*, analysis of cells collected *ex vivo* from infected cattle revealed that virtually all of the infected cells were $CD4^+$ or $CD8^+$ T cells (Emery *et al*. 1988). Moreover, inoculation of calves with comparable numbers of autologous purified T or B lymphocytes incubated *in vitro* for 48 h with *T. parva* sporozoites demonstrated that B lymphocytes produced mild self-limiting infections whereas T lymphocytes (either $CD4^+$ or $CD8^+$) produced severe, potentially fatal infections (Morrison *et al*. 1996). The findings indicated that this was not due merely to a difference in the numbers of infected cells administered but rather reflected the properties of the different cell types. Further studies revealed that cloned *T. parva*-infected T cell lines maintained in culture for 8 weeks or more also exhibited reduced virulence when administered to autologous animals (Morrison *et al*. 1996). These observations demonstrated that virulence of the parasite is determined not just by its ability to transform the host cells but also other phenotypic properties of the infected cells. Since some populations of *Bos indicus* cattle are less severely affected by infection with *T. parva* and *T. annulata* than *Bos taurus* (Glass *et al*. 2005; Ndunga *et al*. 2005), it is also clear that host genotype influences severity of disease. This is further supported by the finding that *T. parva* isolates of buffalo origin transform buffalo cells with similar or greater

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

frequency than bovine cells, but are non-pathogenic in buffalo (Baldwin *et al*. 1986). Although the basis of these differences has not been pursued, these systems offer considerable potential for future studies to define the molecular determinants of virulence.

SUCCESSFUL IMMUNIZATION WITH LIVE PARASITES

The research approaches used to develop methods of immunization against Theileria parasites, including the merits and disadvantages of the different types of vaccines, have been the subject of a recent detailed review (Morrison and McKeever, 2006). Herein, I will focus on the key scientific advances that have underpinned these approaches.

Studies in South Africa in the early 1900s had demonstrated that cattle could be immunized against *T. parva*, albeit inconsistently, by administration of large numbers of cells from lymphoid tissues of animals suffering from East coast fever. Remarkably, this method of immunization was tested in a field trial involving over 280 000 cattle, but unfortunately 25% of the animals died as a result of the immunization procedure, although about 70% of the survivors were immune (Spreull, 1914). The development of methods for cryopreservation of tick-derived sporozoites in the early 1970s provided a means of establishing reference stocks of parasites of known infectivity, which allowed renewed investigation of immunization with live parasites (Cunningham *et al*. 1973, 1974). A series of studies conducted by Cunningham and colleagues demonstrated that infection of cattle with a defined dose of *T. parva* sporozoites and simultaneous administration of a slow release formulation of oxytetracycline (so-called infection and treatment) resulted in mild infection and generation of immunity against subsequent parasite challenge (Radley *et al*. 1975*a*). However, although animals immunized with one parasite isolate were solidly immune to challenge with the same isolate, they exhibited inconsistent protection against heterologous isolates, some animals being solidly immune whereas others remained fully susceptible. Nevertheless, by using a combination of 3 parasite isolates, these workers were able to generate immunity that was effective against experimental challenge with a range of parasite isolates and also against field challenge (Radley *et al*. 1975*b*; Uilenberg *et al*. 1976). Uptake of this vaccine was initially hampered by logistical issues relating to production and distribution, as well as reluctance by some countries to use live parasite strains that may not be present in local tick populations. More recently, the vaccine has been used successfully in several regions of eastern Africa and a new initiative to support commercial production and more widespread use of the vaccine has been funded by the Gates Foundation.

Around the same time that vaccination by infection and treatment was being developed for *T. parva*, a method of vaccination against *T. annulata*, based on the use of cell lines in which the parasite had been attenuated by prolonged passage *in vitro*, was developed (Pipano and Tsur, 1966). Although, again, the resultant immunity was more effective against the homologous parasite isolate, single parasite isolates could be used to vaccinate cattle in the field (Pipano, 1989). This method of immunization has been used locally with success in a number of countries since the 1970s. In each case, a vaccine utilizing a locally derived parasite isolate was used because of concern about introducing 'foreign' parasites into the local tick populations.

In addition to their important contributions to the control of the diseases, these methods of immunization provided highly reproducible experimental systems to study the mechanisms of immunity against the parasites.

DEMONSTRATION OF THE IMPORTANCE OF T CELL RESPONSES IN IMMUNITY

Despite the efficacy of live Theileria vaccines, the use of subunit vaccines would offer potential practical advantages for commercial production and distribution. To this end, research over the last 3 decades has investigated the mechanisms and antigenic specificity of immunity against these parasites.

In 1981, Emery demonstrated that calves could be adoptively immunized against *T. parva* by transfer of thoracic duct lymphocytes from immune to naïve twin calves (Emery, 1981). Lymphocytes collected 6–8 weeks after immunization by infection and treatment and transferred into the naïve co-twin immediately prior to challenge gave substantial protection. Around the same time, Theileria-infected cells were shown to elicit T proliferative responses *in vitro* in autologous lymphocytes from *T. parva*-immune calves (Pearson *et al*. 1979). These cultures were shown to have genetically-restricted cell-mediated cytotoxic activity against parasitized cells and similar cytotoxic activity was also detected in blood lymphocytes harvested from cattle undergoing immunization or challenge with *T. parva* (Emery *et al*. 1981; Eugui and Emery, 1981). The subsequent development of markers for subsets of bovine lymphocytes and reagents for characterizing bovine MHC molecules led to the identification of the effector cells as CD8 T cells restricted by class I MHC (Morrison *et al*. 1987). These and subsequent studies demonstrated that Theileria-infected leukocytes are highly effective antigen-presenting cells for the *in vitro* activation of parasite-specific T cells, of both the CD4 and CD8 lineages. This property allowed the development of systems for *in vitro* maintenance and cloning of parasite-specific T cell lines, which have been used extensively for dissecting

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

the specificity of the T cell response (Goddeeris *et al*. 1986; Taracha *et al*. 1992). Studies of the kinetics of *in vivo* T cell responses in animals undergoing immunization or challenge with *T. parva* had demonstrated the emergence of strong parasite-specific CD8 T cell responses coinciding with remission of infection. Further adoptive transfer experiments carried out in the early 1990s showed that immunity could be transferred between twin calves with lymphocyte populations enriched for CD8 T cells but not with populations depleted of CD8 T cells (McKeever *et al*. 1994), thus providing unambiguous evidence that $CD8^+$ T cells are important mediators of immunity. However, since activated T cells were used in these experiments the findings did not exclude the possibility that other cell types, particularly CD4 T cells, are required for efficient induction and/or recall of the CD8 T cell response. This notion was supported by the subsequent observation that CD4 T cells from immune animals were required for optimal activation of *T. parva*-specific memory CD8 T cells *in vitro* (Taracha *et al*. 1997).

It is of note that parasite-specific CD8 T cell responses are not detected until 14–18 days after immunization by infection and treatment. Nevertheless, primary infections with *T. parva* and *T. annulata* result in vigorous T cell responses, including a large component of CD8 T cells, 7–10 days after infection (Nichani *et al*. 1998; Houston *et al*. 2008). However, these T cells differ in phenotype and function from those in immune animals, most notably in the absence of specific cytotoxic activity for parasitized cells. Based on these observations, it has been proposed that infection induces dysregulation of the primary T cell response resulting in delayed parasite clearance (Houston *et al*. 2008).

SPOROZOITES AS AN ALTERNATIVE TARGET FOR VACCINATION

In the early 1980s, monoclonal antibodies generated against the sporozoite stages of *T. parva* and *T. annulata* were shown to neutralize the infectivity of sporozoites for leukocytes *in vitro* (Dobbelaere *et al*. 1984, 1985; Musoke *et al*. 1984; Williamson *et al*. 1989). In each case, the antibodies recognized a dominant antigen of between 60 kDa and 70 kDa (known as p67 in *T. parva* and Spag in *T. annulata*) (Nene *et al*. 1992; Hall *et al*. 1992), located within a loosely arranged proteinaceous coat on the surface of the sporozoite (Webster *et al*. 1985). Binding of the antibodies was shown to prevent invasion of the host cell by the sporozoites. Cattle exposed to a single infective dose of *T. parva* sporozoites, such as that used for infection and treatment, showed little or no anti-p67 antibodies (Musoke *et al*. 1984), probably reflecting the small quantities of sporozoite antigen to which they are exposed. However, repeated challenge of immune animals with sporozoites was shown to result in induction of higher levels of antibodies capable of neutralizing the infectivity of sporozoites *in vitro* (Musoke *et al*. 1982). These observations led to a series of studies aimed at investigating the possibility of using these antigens for vaccination. Recombinant versions of the proteins, produced in *E. coli* or baculovirus, administered in adjuvant were shown to induce high titres of neutralizing antibodies but only resulted in protection against challenge in a proportion of the immunized animals (Musoke *et al*. 1992; Hall *et al*. 2000; Kaba *et al*. 2005). The baculovirus-expressed *T. parva* p67 protein appeared to give protection in a larger proportion of the immunized animals than the *E. coli*-expressed version (Kaba *et al*. 2004), but immunization with the two forms of the protein was not compared in the same experiment. Intriguingly, in many of these studies neither the levels of neutralizing activity nor the epitope specificity of the antibody in individual animals appeared to correlate closely with protection, suggesting that the immunity might not be attributable solely to the antibody response. A field vaccine trial using recombinant *T. parva* p67 expressed in *E. coli* resulted in protection in some animals (Musoke *et al*. 2005) but the level of protection was not sufficient to justify commercial development. These findings illustrate the difficulty of achieving robust immunity against this early stage of the parasite (reviewed by Morrison and McKeever, 2006). Titrations of infectivity of *T. parva* in cattle suggest that establishment of infection in a few thousand, or perhaps a few hundred, cells is sufficient to produce clinical disease (Cunningham *et al*. 1974). Hence, complete or near complete blockade of infection may be required to achieve immunity, in the absence of protective activity against subsequent parasite stages. Nevertheless, these antigens may eventually prove to be useful components of vaccines incorporating antigens from different stages of the parasites.

PARASITE STRAIN SPECIFICITY OF THE CD8 T CELL RESPONSE CORRELATES WITH IMMUNITY

Given the evidence that CD8 T cells play a key role in immunity, an obvious question to ask was whether parasite strain specificity of the CD8 T cell response was responsible for the observed incomplete cross-protection between parasite isolates. A number of studies involving analyses of $CD8^+$ T cell responses in animals immunized with the Muguga isolate of *T. parva* demonstrated that the $CD8^+$ T cells recognized cells infected with some heterologous parasites but not others (Goddeeris *et al*. 1986, 1990). Moreover, the pattern of strain specificity of the responding CD8 T cells varied among animals immunized with the same parasite isolate and this

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

variation was shown to be determined, at least partly, by the MHC genotype of the host (Goddeeris *et al.* 1990; Taracha *et al.* 1995*b*). These studies also revealed differential CD8 T cell recognition of cloned parasitized cell lines derived from the same parasite isolates. Analysis of these isolates with DNA probes confirmed that they were genetically heterogeneous (Goddeeris *et al.* 1990). This heterogeneity complicated the interpretation of experiments to examine the relationship of CD8 T cell specificity and cross-protection between parasite isolates. The production of cloned sporozoite stocks, by tick pick-up of infections initiated with cloned autologous parasitized cell lines, overcame this problem (Morzaria *et al.* 1995). A detailed analysis of the specificity of CD8 T cell responses undertaken in animals immunized with the Muguga isolate or a cloned derivative of the Marikebuni isolate and subsequently challenged with the reciprocal parasite, provided clear evidence that the specificity of the response induced by immunization correlated with susceptibility to heterologous parasite challenge (Taracha *et al.* 1995 *a*, *b*). Thus, animals that generated CD8 T cell responses reactive with the two parasites were solidly immune to the heterologous parasite, whereas animals that had responses specific for the immunizing parasite developed clinical reactions of varying severity following challenge. Although the Muguga parasite used in these studies was uncloned, there was evidence that this isolate was antigenically homogeneous and subsequent genetic analyses have shown that it varies at only 3 of 81 polymorphic DNA loci (F. Katzer, personal communication). The results of these studies provided further evidence that CD8 T cell responses play an important role in immunity and also indicated that the antigenic specificities detected by the cytotoxicity assays employed are likely to be targets of the protective response.

CONTRIBUTION OF PARASITE GENOME SEQUENCES

The recent generation of complete genome sequences for *T. parva* and *T. annulata* (Gardner *et al.* 2005; Pain *et al.* 2005) has been an important milestone that has opened up a number of important new research opportunities. Previous studies had revealed that these parasites had relatively compact genomes in comparison to other apicomplexan parasites, identifying 4 chromosomes and providing estimates for genome size of <10 million base pairs (bp). The genome sequences confirmed the chromosomal organization and showed that both parasites had a genome of approximately 8·3 million bp with around 4000 annotated protein-encoding genes. The two genomes showed a high level of synteny across all chromosomes and over 80% of the identified genes were orthologous between the two species. Subsequent transcriptional analyses of *T. parva* have indicated that over 60% of the identified genes are expressed in the intra-lymphocytic schizont stage of the parasite (Bishop *et al.* 2005). In general, the genomes contained fewer repetitive sequences and multi-member gene families than most of the other apicomplexan genomes, although one novel family of genes (the subtelomere-encoded variable secreted proteins – SVSP), located in tandem arrays in the subtelomeric regions of all chromosomes, was identified in both parasites. Eighty-five of these genes were identified in *T. parva*; they have predicted signal peptides and have been shown to be expressed in a small percentage of the cells in established cell lines, but their function is as yet unknown (Schmuckli-Maurer *et al.* 2009). The genome sequences, as well as whole genome arrays generated from the sequences, are now key resources that underpin current research on these parasites.

IDENTIFICATION OF ANTIGENS RECOGNIZED BY PARASITE-SPECIFIC T CELLS

Until recently, further progress in understanding the antigenic specificity of immunity to *T. parva* had been hampered by difficulties in identifying target parasite antigens. This obstacle has been overcome with the recent development, by workers at the International Livestock Research Institute (ILRI) in Kenya, of high throughput systems for antigen screening. Two methods were used, both based on measurement of IFN-γ production by parasite-specific CD8 T cells following incubation with COS-7 cells co-transfected with parasite cDNAs and class I MHC heavy chains. The first involved testing individual candidate genes selected from the genome sequence based on possession of a predicted signal peptide. The second utilized a cDNA library prepared from purified schizonts, from which a series of pools of 50 clones were initially tested and positive pools then resolved by further testing of the constituent clones. This latter approach was based on a screening system initially developed and used successfully for identification of antigens recognized by human tumour cell-specific $CD8^+$ T cells (DePlaen *et al.* 1997).

Using these methods, Graham and colleagues (2006, 2007) identified 6 *T. parva* antigens recognized by parasite-specific $CD8^+$ T cell lines, and several additional antigens have been identified in subsequent work. The antigens are encoded by unrelated genes distributed across 3 of the parasite chromosomes. A striking feature of the results of antigen screening was that CD8 T cell lines from cattle of different class I MHC genotypes tended to identify different target antigens. Studies in which the epitopes presented by a number of defined class I MHC alleles were identified, revealed a single dominant epitope (9–11 amino acids) for all but 1 of

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

the antigen-MHC allele combinations (Graham *et al.* 2008). These findings supported earlier suggestions, based on analyses of strain specificity of CD8 T cell responses, that the response to *T. parva* in animals of different MHC genotypes is focused on different antigenic specificities.

Analysis of sequence data for 2 of the antigens (Tp1 and Tp2) in a sample of 27 isolates of *T. parva* has identified 2 variant sequences for each antigen. The sequence variation resulted in amino acid substitutions within the defined T cell epitopes in both antigens and functional testing of these variants confirmed that the substitutions result in loss of recognition by CD8 T cell clones raised against the native epitope (MacHugh *et al.* 2009). The Tp2 gene exhibited a particularly high level of sequence diversity, both variants showing identities of only about 75% with the Muguga genome sequence and containing 4–6 predicted amino acid substitutions in each of the two CD8 T cell epitopes examined. These studies are being extended to the other antigens using a larger panel of parasites, including isolates from buffalo (R. Pelle, personal communication). Given that *T. parva* is likely to have originated in buffalo and that many buffalo parasites do not appear to transmit between cattle, the buffalo *T. parva* population may contain greater antigenic diversity than that maintained in cattle. In this regard, it is of interest that the p67 sporozoite surface antigen shows a limited degree of polymorphism in buffalo-derived isolates but is apparently conserved in the cattle-maintained population of *T. parva* (Nene *et al.* 1996).

IMMUNODOMINANCE AND GENETIC EXCHANGE AS DETERMINANTS OF PARASITE STRAIN RESTRICTED IMMUNITY

The phenomenon of immunodominance, whereby the T cell response is focused on a few of the antigens to which an animal is capable of responding, is a well-recognized feature of CD8 T cell responses to many virus infections (Yewdell, 2006). Observations from studies of the strain specificity of responses to *T. parva* indicated that immunodominance is also a feature of CD8 T cell responses to this parasite. Thus, some animals immunized with one parasite isolate and subsequently challenged with a second isolate were found to generate strain restricted CD8 T cell responses following immunization but produced CD8 T cells reactive with both parasites following challenge, suggesting that there is a hierarchy of antigenic dominance (Taracha *et al.* 1995*b*). Profound immunodominance of the response has been confirmed in recent experiments involving detailed clonal analysis of CD8 T cell responses in cattle homozygous for the A18 and A10 MHC haplotypes (MacHugh *et al.* 2009). These animals generated detectable CD8 T cell responses to only 1 of 5 antigens tested, Tp1 in A18+ animals and Tp2 in A10+ animals, and in each case over 70% of the responding T cells were found to be specific for the single dominant antigen. Importantly, comparison of the expressed T cell receptor (TCR) β chain variable genes expressed by *in vitro* T cell cultures and T cells harvested *ex vivo* from the same animals confirmed that the CD8 T cell lines used for these analyses were indeed representative of the *in vivo* memory populations (Connelley *et al.* 2008; MacHugh *et al.* 2009). These findings provided confirmatory evidence that the CD8 T cell response induced by immunization with a single parasite strain is highly focused on dominant antigens, which differ depending on the host MHC genotype, and indicated that the strain specificity of the response will be dictated by the nature and extent of polymorphism of these antigens.

The known capacity of apicomplexan parasites to undergo sexual recombination has the potential to generate a further level of complexity in antigenic diversity. Early morphological observations provided evidence of a sexual stage of development in *T. parva* in the tick vector (Melhorn and Schein, 1984). More recently, experimental studies have confirmed the occurrence of genetic recombination between different parasite strains and illustrated its capacity to generate a high level of genetic diversity (Katzer *et al.* 2006). However, the importance of genetic exchange in shaping the genotypic and antigenic make-up of field populations depends on the frequency with which it occurs in natural settings. The genome sequence for *T. parva* has been exploited to identify over 80 satellite and other polymorphic DNA markers for genotyping parasite populations (Oura *et al.* 2003; Katzer *et al.* 2006). A series of studies of populations of *T. parva* in different regions of Uganda and Kenya, using a subset of these markers, have revealed a high level of diversity and a high frequency of infection of cattle with mixed genotypes (Oura *et al.* 2005, 2007; Odongo *et al.* 2006). Although, some evidence of geographical substructuring was found within the parasite population, analyses of the genotypes indicated that there is frequent genetic exchange. Consequently, parasite populations, although genotypically diverse, are likely to show complex patterns of antigenic relatedness. I have proposed that this population structure, coupled with focusing of the CD8 T cell response on dominant polymorphic antigens, accounts for the observed patterns of incomplete cross-protection between parasite strains (Morrison, 2007). Although genetic exchange can generate enormous genotypic diversity, it may (depending on the extent of polymorphism in the antigens) increase the likelihood that any pair of parasite clones will share alleles of some of the dominant antigens. Hence induction of CD8 T cell responses to a larger number of antigens in individual animals, even though the antigens are polymorphic, may be

https://doi.org/10.1017/S0031182009990916 Published online by Cambridge University Press

sufficient to provide broad protection. This may account for the ability to generate broad protection with the 3 parasite isolates used in the infection and treatment vaccine, if each of the component parasites induces its own CD8 T cell response.

CONCLUDING REMARKS

Despite the small size of the Theileria research community, in comparison with those working on other important pathogenic parasites, substantial progress has been made in the last 40 years in understanding the cell biology of the parasites and the nature of host protective immune responses. The findings have revealed an intricate relationship between Theileria parasites and their host cells, which allows rapid parasite multiplication and survival in the face of host cellular responses. Major progress has been made in understanding the protective immune responses and the antigenic basis of the observed incomplete cross-protection between parasite strains. The recent generation of parasite genome sequences has been an important step that has allowed rapid progress in antigen identification and has led to the production of a range of molecular tools for analysis of parasite population diversity. These advances provide exciting opportunities for research into vaccine development. In contrast to the situation 40 years ago, this improved knowledge base now allows us to define more precisely the scientific questions that need to be addressed in taking this work forward.

ACKNOWLEDGEMENTS

Some of the work discussed in this paper was supported by Wellcome Trust grant No. 075820.

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