Protective immunity against *Trypanosoma cruzi*

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Upon infection, *Trypanosoma cruzi* triggers a strong immune response that has both protective and pathological consequences. In this work, several important questions regarding protective immunity are reviewed. Emphasis is placed on recent studies of the important protective role of CD8+ T cells and on previous studies of immunisation of domestic *T. cruzi* reservoirs that sought to address practical vaccination problems. Research on the maturation of memory cells and studies indicating that the prevalence of *T. cruzi*-specific T-cell responses and a high frequency of committed CD8+ T cells are associated with better clinical outcomes are also reviewed. Additionally, animal models in which protection was achieved without immunopathological consequences are discussed.

Key words: *Trypanosoma cruzi* - immunity - CD8+ T cells

Clinical studies indicate that *Trypanosoma cruzi* infection results in a state of host resistance that controls parasite load after one to three months. Parasitemia levels are high during the first few weeks of infection but decrease thereafter to almost undetectable levels, as indicated by parasite detection using fresh blood mounts, hemocultures, xenodiagnosis or polymerase chain reaction (PCR). Evidence that an otherwise progressive and lethal infection is usually suppressed by the immune system is provided by cases of reactivation or appearance of atypical clinical presentations of acute *T. cruzi* infection in patients with acquired immunodeficiencies (Simao Ferreira 1999) or human immunodeficiency virus infections (Karp & Arwaerter 2007).

Experimental mouse models, in which primary and secondary inoculations with *T. cruzi* may be planned at desired sequences and intervals, have been used to demonstrate that control of parasite load and survival during the chronic phase are the effects of a general protective mechanism elicited by primary infection. When chronically infected mice were again challenged with virulent *T. cruzi*, they do not exhibit a pathological response, whereas naive control mice experience high parasite load and high mortality (Basombrio 1981). For many years, inoculations with an attenuated *T. cruzi* culture (TCC) strain, containing mostly epimastigote forms, provided our laboratory with a method of reliably inducing protective immunity. This technique resulted in stronger immunity for a longer duration than either selected recombinant *T. cruzi* antigens plus adjuvants or killed parasites. Although this method is not approved for human vaccination trials, it has allowed us to study practical questions pertaining to vaccination against *T. cruzi* in laboratory animals and domestic reservoirs.

Here, we review several important questions regarding protective immunity against *T. cruzi*. Although there is a vast literature on the subject, we place emphasis on experiences from our laboratory. Many of these research questions are longstanding but have been reformulated as technical progress provides new perspectives.

Have animal models of Chagas disease contributed evidence for or against the theory of autoimmune pathogenesis?

It is tempting to hypothesise that the immune response itself causes disease. From this perspective, one would expect to detect pathology when the immune system is actively suppressing *T. cruzi* load. A first series of experiments in our laboratory used the virulent Tulahuen strain of *T. cruzi*, later identified as *T. cruzi* Ile (TCIIe), to induce primary and secondary infections in mice (Table). Blood parasite load and mortality were clearly reduced after the second challenge, but the mice nevertheless developed severe heart and muscle lesions (Basombrio 1981). Studies using the *T. cruzi* I (TCI) lineage provided findings consistent with these results, showing that some TCI strains induced serious tissue lesions despite minimal or undetectable parasitemia (Cardozo 2003). However, in several experiments in which a highly attenuated primary infection by the TCC strain (TCI lineage) (Diósquique et al., unpublished observations) preceded a virulent TCIIe challenge, many desirable vaccination effects were demonstrated without immunopathology (Basombrio et al. 1982). Taken together, these studies indicate that the relationship between parasite load, inflammation and pathology in *T. cruzi* infection is highly dependent on the lineage of the infecting parasite and that protective immunisation does not necessarily trigger an immunopathological response. Therefore, immunisation with TCC parasites confers protection against re-challenge and does not lead to immunopathology, which would be expected in an autoimmune context.
What is the role of CD8+ T cells in Chagas disease?

*T. cruzi* is an intracellular pathogen that replicates in the cytoplasm of a wide variety of mammalian host cells, including macrophages and cardiac myocytes. Robust CD4+ and CD8+ T-cell responses are needed to protect against this protozoan, since mice lacking either of these T-cell subsets succumb to infection (Tarleton et al. 1992, 1996, Padilla et al. 2007). In particular, CD8+ T cells are a critical component of protective immunity in both mouse models and human patients (Alvarez et al. 2008). The significant role of CD8+ T cells during *T. cruzi* infection is demonstrated by their predominance in non-lymphoid tissues not only in experimental models, but also in the myocardium of *T. cruzi*-infected cardiac patients (Vogt et al. 2008). CD4+ and CD8+ T cells also secrete interferon-γ (IFN-γ), which is crucial for activating macrophages that exert trypanocidal activity via nitric oxide (Muñoz-Fernandez et al. 1992).

Although CD8+ T cells mount a substantial immune response against *T. cruzi* infection, the parasite may survive and establish a lifelong chronic infection in the host (Martin & Tarleton 2006, Tzepelis et al. 2006). This parasitic persistence contributes to the development of symptoms many years or decades after initial infection in approximately one-third of chronically infected individuals (Tarleton 2001, 2003).

What are the contributions of experimental mouse models to the analysis of T-cell responses to persistent or resolved *T. cruzi* infections?

When *T. cruzi* infection occurs, the innate and acquired immune responses are characterised by the recruitment of dendritic cells, macrophages, natural killer (NK) cells, and B and T lymphocytes, as well as their secretion of soluble factors (cytokines and chemokines). Once parasite load is controlled in chronically infected mice, the majority of *T. cruzi*-specific CD8+ T cells display a T effector or T effector memory (TEM) phenotype (CD62Llo, CD122lo, CD127lo). This phenotype correlates with a late-differentiation pattern and its maintenance has been thought to be antigen-dependent (Martin & Tarleton 2005). However, after pathogen clearance by drug treatment, long-term exposure to *T. cruzi* does not exhaust pathogen-specific CD8+ T cells, which maintain their potential to address re-infection (Bustamante et al. 2008). In this case, the development of a stable, antigen-independent TEM (T central memory) cell population (CD62Lhi, CD122lo, CD127lo, CCR7lo, and increased Bcl-2 expression) has been noted. This population provides better protective immunity upon re-challenge than T effector or TEM cells in chronically infected mice. The possibility that phenotypic reversion occurs after parasitic load is decreased has important implications in human disease, as it may represent a new means of assessing treatment efficacy.

The influence of parasite persistence on the immune response to *T. cruzi* has also been studied in our laboratory. We hypothesised that if a memory mechanism operates in the absence of infection, long-term resistance to virulent reinoculation should persist regardless of whether the primary infection was cured. In support of this hypothesis, treatment of TCC-inoculated mice with benznidazole did not terminate resistance to virulent inocula administered as late as 330 days after primary infection (Basombrio et al. 2002b). However, TCC inoculation of drug-treated mice did diminish their long-term (1 year) ability to resist secondary challenge. These observations suggest that both parasite persistence and immunological memory contribute to long-term resistance to re-infection.

Is there a relationship between disease progression and the pattern of T-cell differentiation in *T. cruzi*-infected humans?

During persistent human viral infection, antigen-primed CD8+ T cells may be classified as having early, intermediate and late-differentiated phenotypes based on their sequential down-regulation of CCR7, CD28, and CD27 and up-regulation of cytotoxic factors (Appay et al. 2002; Appay & Rowland-Jones 2004). Protective immunity is best conferred by early-differentiated T cells, which possess potent proliferative capacity (Appay & Rowland-Jones 2004). Meanwhile, highly differentiated T cells present signs of senescence during chronic infection, presumably due to exhaustion after persistent activation and restimulation (Barber et al. 2006, Hinrichs et al. 2006).

In chronically *T. cruzi*-infected patients presenting severe disease, the phenotypic profile of the antigen-experienced memory CD8+ T-cell population is enriched in fully differentiated memory CD45RAlo, CD27lo, CD28lo CD8+ T cells (Laucella et al. 2004). Similarly, a low frequency of *T. cruzi*-specific CD8+ T cells has been observed in these patients, perhaps due to less efficient generation of memory CD8+ T cells or more marked decline in memory T-cell function over time. This apparent impairment of CD8+ T-cell responses is associated with an increased frequency of fully differentiated memory CD8+ T cells and a high apoptosis rate, possibly reflecting CD8+ T-cell exhaustion (Alvarez et al. 2008). Conversely, more frequent *T. cruzi*-specific T-cell responses

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PalDal(I): a *T. cruzi* isolate; TCC (I): *T. cruzi* culture I; Tul (Ile): Tulahuen Ile; ++: moderate; +++: severe; ±: slight; -: not detectable.

TABLE Summary of studies on parasite load and histopathology after sequential inocula of Trypanosoma cruzi in Swiss mice

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are associated with better clinical outcomes, suggesting that protection from infection is related to an increased prevalence of highly competent, early-differentiated CD8+ T cells, which has been observed in patients with mild or no disease symptoms (Albareda et al. 2006).

Is protective immunity capable of completely preventing re-infection?

Vaccine testing in mice is primarily composed of measurements of acute-stage parasitemia and mortality. Some studies have adopted more sensitive determinations of parasitemia, including hemoculture (Machado et al. 2006), xenodiagnosis (Basombrio et al. 1986, Basso et al. 2007), histopathological studies (Basombrio & Besuschio 1982, Basombrio et al. 1986, Machado et al. 2006, Hoft et al. 2007) and electrocardiographic measurements (Cuneo et al. 1989). However, perhaps the most stringent method of demonstrating the absence of infection or “sterile immunity” after inoculation is serological testing. To explore this possibility, we applied the complement-mediated lysis (CML) test developed by Krettli and Brener (1982) to a mouse model of T. cruzi infection that included wild T. cruzi isolates as well as TCC and Tulahuen strains. While many animals appeared protected from re-infection based on several parasitemia detection tests, sub-patent infections were demonstrated by the presence of lytic antibodies. Nevertheless, a significant proportion of mice (8/18 for wild T. cruzi infection, 25/28 for Tulahuen infection) lacked lytic antibodies, suggesting a complete absence of re-infection in TCC strain-immunised mice (Gomez et al. 1996). The CML test demonstrated infection by the Tulahuen strain and wild isolates but not by the TCC strain, which, besides being attenuated, did not elicit lytic antibodies. However, recent experiments have demonstrated TCC parasite persistence by hemoculture and real-time PCR after immunosuppression of mice infected with TCC trypanomastigotes from tissue cultures (Padilla et al., unpublished observations). These results suggest that protective immunisation against T. cruzi can prevent re-infection after secondary challenge, although this strong immunity may be induced by a sub-patent, immunizing infection. Field trials in dogs included negative indirect hemagglutination (IHA) tests that indicated the absence of infection in immunised animals (Basombrio et al. 1989). The CML and IHA tests are based on distinct reactions, the latter has also been used to infer therapeutic success in clinical settings.

Can the prevention of infection be gauged by parameters indicative of disease?

In “premunition” experiments in mice challenged with the Tulahuen strain TCCIIe, re-infection lethality and blood parasite load were strongly suppressed. However, these mice suffered from severe muscle, intestine and heart histopathological lesions (Basombrio 1981). Using the same experimental design but replacing the Tulahuen strain with the attenuated TCC strain during primary infection, lethality and parasite load were again suppressed during secondary infection. Additionally, many measurable parameters of histopathological damage, including inflammatory mononuclear infiltration, necrosis and fibrosis, indicated protective immunity without immunopathological side effects (Basombrio et al. 1982, Basombrio & Besuschio 1982).

In another experiment, the frequency of certain electrocardiographical alterations, such as persisting atrioventricular blocks, supraventricular tachycardia and sinus bradycardia was measured in T. cruzi-infected mice previously or never primed with TCC strain inocula (Fig. 1). The TCC-primed group displayed a significant reduction in electrocardiographic alterations (Cuneo et al. 1989). Pathology of heart and skeletal muscle, tissue parasite load and inflammation, mortality and parasitemia were also reduced in T. cruzi-infected mice previously immunised with DNA vaccines containing the trans-sialidase family genes ASP-1, ASP-2, or TSA-1 (Garg & Tarleton 2002, Vasconcelos et al. 2004).

Do the time intervals between immunisation and infection in experimental models properly mimic those of natural T. cruzi infection in the field?

Unfortunately, although the duration of protective immunity development is an important parameter, it is tested and reported very infrequently for T. cruzi vaccines. The fact that protection by non-replicating T. cruzi antigens is relatively short-lived (1-3 months) unless booster injections are provided probably precluded the investigation of many candidate immunogens. In this regard, the vaccination experiments of Machado et al. (2006), which used recombinant adenoviruses expressing two T. cruzi antigens in a replicating organism, are very promising, particularly since protection against re-infection was demonstrable 3 1/2 months after immunisation. Moreover, many of the immunised mice developed sterile immunity, as suggested by negative hemocultures, and all protected mice survived a 100% lethal challenge.

Tzelepis et al. (2006) studied the kinetics of the CD8+ T-cell cytotoxic response and IFN-γ secretion after in-
fection of naive mice or mice vaccinated with trans-sialidase or ASP-2 antigens. Following peak parasitemia, these responses reached a maximum around days 15-30 of infection, subsequently declining slowly but still detectable by day 240. In particular, cytotoxic activity against ASP-2 peptide-coated cells was remarkably high eight months after initial infection. While the cytotoxic response peaked after the highest parasitemia level in naive mice, vaccinated mice responded much more rapidly, leading to more successful protection. Inoculation with the TCC strain induces lifetime resistance to reinfection with virulent wild T. cruzi isolate, as tested with inocula administered 312, 366 or 436 days after primary TCC infection (Basombrio & Arredes 1987). Thus, the duration of the development of protective immunity prior to re-infection seems essential to vaccination in the field. The importance of this time interval between primary and secondary infection in the field may explain why killed T. cruzi vaccines that were highly protective in laboratory tests were only weakly protective in field tests on guinea pigs (Basombrio 1990).

Is the acquired immunity demonstrated in immunised laboratory mice also operative against natural reservoirs of T. cruzi, such as dogs and guinea pigs infected with wild T. cruzi by insect vectors?

Most testing of experimental T. cruzi vaccines has been performed under laboratory conditions, which may differ significantly from a field setting. Bloodborne trypanastigotes of a laboratory T. cruzi strain are typically injected intraperitoneally or subcutaneously into mice after a short, uniform vaccination-challenge interval. In contrast, a field test of the same vaccine requires vectorial inoculation of insect-borne, wild forms of the parasite at mucosal or intradermal sites in mammals known as natural T. cruzi reservoirs. This protocol also includes relatively long and highly variable vaccination-challenge intervals.

One approach to testing immunity against “natural” infection modalities is oral delivery of vector-borne trypastigotes in mice, allowing exploration of the particular effectors of mucosal immunity. For example, vaccines composed of recombinant trans-sialidase and the adjuvant oligodeoxynucleotide CPG-ODN were found to be effective, even when administered intranasally, against secondary oral challenge with T. cruzi (Hoft et al. 2007).

Guinea pigs, which are both domestic reservoirs of T. cruzi and a convenient experimental model, gave us the opportunity to perform vaccination studies in isolated field yards colonised by Triatoma infestans vectors. TCC parasite cultures used in a live-attenuated vaccine were shown to be unable to propagate outside culture or to feed the natural transmission cycle. After five replications of the experiment, the rate of natural infection, as tested by xenodiagnosis, had been reduced to 39% in animals preinoculated with 10’ TCC culture forms, as compared with 63% in control guinea pigs (p < 0.02) (Fig. 2). After publication of these results (Basombrio et al. 1987), 10 additional experimental series involving a total of 149 control and 157 vaccinated guinea pigs demonstrated a significant degree of protection in the latter group, with infection rates reaching 57.7% and 36.3%, respectively (p < 0.001).

Following pioneering work conducted in Brazil (Mezeis 1969), we attempted experimental vaccination in dogs. Like guinea pigs, dogs are important domestic reservoirs of T. cruzi, displaying high rates of natural infection in some subtropical areas of the Santiago del Estero province of Argentina. In this region, a house-to-house survey allowed the selection of 219 uninfected animals, which were randomised either to be vaccinated with two inoculations of TCC live-attenuated cultures or to remain untreated (Basombrio et al. 1993). After one year of natural exposure to vectors, T. cruzi infection was examined by means of xenodiagnosis and serological hemagglutination reactions in 148 dogs. It was found that the natural T. cruzi infection rate, determined using
the control group (20/75; 26.7%) had been reduced (9/73; 12.3%) in the vaccinated animals (p < 0.015) (Fig. 3). This trial indicated that natural *T. cruzi* infection of dogs is an epidemiological condition that may be improved by vaccination, which may help to reduce the risk of spreading infection to humans.

**Can measurements of parasite trafficking in the transmission cycle inform assessment of protective immunity?**

Several successful vaccines exert a “herd effect” in addition to protecting the individual, whereby transmission of the target pathogen from vaccinated to non-vaccinated hosts is partially blocked. Theoretically, this effect may be measured for the complex host-vector transmission cycle of *T. cruzi* in two ways. Host-to-vector traffic can be correlated to the host’s ability to transfer the parasite to uninfected insect vectors. This measurement may be obtained by using xenodiagnosis to quantify the proportion of insects infected after a standardised blood meal from a single host. Meanwhile, vector-to-host traffic can be correlated to the ability of insect vectors to transmit the infection to new hosts. This measurement can be obtained by determining the number of bites necessary for infection (NBNI), a calculation applicable to *T. cruzi* transmission in standardised guinea pig yards (Basombrio et al. 1996).

Host-to-vector traffic was measured during several experiments in which guinea pigs living in *T. infestans*-colonised yards were vaccinated with live-attenuated *T. cruzi*. Totals of 2,040 and 1,960 insects were allowed to feed on control and vaccinated guinea pigs, respectively, and were then studied for infection in pools of 10. The positivity rate in xenodiagnosis was 38% for controls and only 18% for vaccinated animals (p < 0.001) (Basombrío et al. 1987).

A similar measurement was performed in vaccinated dogs. Here, the insects were not analysed in pools but individually and xenodiagnoses of uninfected dogs were excluded from the study. Again, the positivity rate was significantly reduced in the vaccinated group, from 52% in control animals to 29% (p < 0.001) (Basombrío et al. 1993). Taken together, the results indicate that in both guinea pigs and dogs subjected to an environment of high vectorial *T. cruzi* transmission, the force of host-to-vector infection was reduced by vaccination. The reduction of *T. cruzi* transmission from dogs to *T. infestans* after experimental vaccination has also been confirmed by Basso et al. (2007).

Vector-to-host *T. cruzi* traffic, as measured by NBNI determinations, was estimated in guinea pig yard experiments. Parameters such as days of exposure, average number of insects per guinea pig, proportion of infected vectors, proportion of fed vectors, number of guinea pigs and proportion of hosts infected were controlled. Whereas an average of 4,973 bites was calculated to infect a control guinea pig, an average of 21,307 bites (a 4.28-fold increase) was necessary to produce the same effect in a vaccinated animal (Basombrío et al. 1997). These results suggest that the effects of protective immunisation on domestic mammalian reservoirs of *T. cruzi* may be measured not only in terms of parasite load, disease and mortality, but also in terms of vectorial parasite traffic (Fig. 4).

**In conclusion**, protective immunity against *T. cruzi* has been well demonstrated in both humans and experimental animal models. However, many escape mechanisms exist whereby parasites may overcome host protective immunity. While *T. cruzi* implements some of these mechanisms, it is unable to generate diverse antigenic lineages or to switch membrane antigens rapidly, which would preclude vaccination. Recent advances toward the development of genetically modified parasites capable of inducing a protective immune response without causing pathology present a promising new ap-
proach to vaccination against *T. cruzi* (Basombrío et al. 2002a, Barrio et al. 2007, Zago et al. 2008). Beyond reducing immunopathology, such a vaccine would potentially establish robust, long-lasting protection without parasite persistence.

Do we need a human *T. cruzi* vaccine at present? The success of well-established vector control and alternative prevention methods indicates that a human vaccine is not imperative but that vaccination of domestic reservoirs may be desirable in some cases of Chagas disease. However, epidemiological situations do change over decades, such as the development of insecticide resistance by vectors or the appearance of new transmission modalities. In these situations, the need for additional prevention methods, including vaccination, may arise.

REFERENCES


