Probing *Trypanosoma cruzi* biology with DNA microarrays

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SUMMARY

The application of genome-scale approaches to study *Trypanosoma cruzi*–host interactions at different stages of the infective process is becoming possible with sequencing and assembly of the *T. cruzi* genome nearing completion and sequence information available for both human and mouse genomes. Investigators have recently begun to exploit DNA microarray technology to analyze host transcriptional responses to *T. cruzi* infection and dissect developmental processes in the complex *T. cruzi* life-cycle. Collectively, information generated from these and future studies will provide valuable insights into the molecular requirements for establishment of *T. cruzi* infection in the host and highlight the molecular events coinciding with disease progression. While the field is in its infancy, the availability of genomic information and increased accessibility to relatively high-throughput technologies represents a significant advancement toward identification of novel drug targets and vaccine candidates for the treatment and prevention of Chagas' disease.

Key words: Host response, trypanosome, microarray, Chagas' disease, differentiation.

INTRODUCTION

The public health burden and economic impact of Chagas' disease in Latin America are significant where more than 10 million people are currently infected with *Trypanosoma cruzi*. One third of chronically infected individuals will develop severe cardiomyopathy and/or digestive disease, the hallmarks of Chagas' disease. No vaccine is available to prevent *T. cruzi* infection and available chemotherapeutic agents are toxic and unable to clear tissue parasites in chronically infected individuals. While coordinated efforts to reduce insect vector transmission in rural domestic settings has had a major impact on the number of acute *T. cruzi* infections in several South American countries (Dias, Silveira & Schofieldi, 2002), there remains a clear need to advance alternate strategies for treatment and control of Chagas' disease (Urbina & Docampo, 2003).

The field of parasitology has embarked on an exciting new era of gene discovery. Genome sequence information is rapidly emerging for many of the major protozoan pathogens. Combined with the availability of annotated human and mouse genomes, a unique opportunity is presented to apply relatively high-throughput, genome-scale methodologies to further our understanding of host-pathogen interactions *in vitro* and in the context of host infection *in vivo*. This is particularly heartening for investigators dedicated to the study of organisms that are less tractable to standard genetic approaches (e.g. genetic screens for virulence determinants) that have been invaluable to the advancement of the bacterial pathogenesis field (Finlay & Falkow, 1997). Even among the trypanosomatids, *T. cruzi* is significantly less compliant than *Leishmania* spp. and *Trypanosoma brucei*, for which a variety of genetic tools are available (Beverley, 2003). However, with sequencing and assembly of the *T. cruzi* genome nearing completion (N. el-Sayed, personal communication), analysis of novel *T. cruzi* genes, patterns of gene expression during development and during host interactions will significantly advance our knowledge of fundamental aspects of *T. cruzi* biology.

To this end, investigators have recently begun to employ genomic approaches to define global host transcriptional responses to *T. cruzi* infection in cultured mammalian cells and in experimental mouse models (de Avalos *et al*. 2002; Ferreira *et al*. 2002; Garg, Popov & Papaconstantinou, 2003; Mukherjee *et al*. 2003). In addition, the first report of the use of DNA microarray technology to analyze stage-specific gene expression on a more global level during the complex *T. cruzi* differentiation process has emerged (Minning *et al*. 2003). These pioneering studies, which are the focus of the present review, reveal novel insights into the biology of the parasite–host interaction and highlight molecular events coinciding with *T. cruzi* developmental changes and disease progression in the host.

T. CRUZI DEVELOPMENTAL CYCLE

During its life-cycle, *T. cruzi* infects both invertebrate and vertebrate hosts where developmental changes are associated with distinct functional roles. In the midgut and rectum of the triatomine vector, non-infective epimastigotes undergo a process of differentiation to produce mammalian-infective

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metacyclic trypomastigotes. Metacyclics are extruded in the insect's faeces as it acquires a bloodmeal and parasites gain access to the vertebrate host through breaches in the skin (e.g. at the bite site) or through the conjunctival mucosa by mechanical introduction (i.e. rubbing parasites into the eye).

The principal role of the non-dividing trypomastigote form of *T. cruzi* (metacyclic or tissue-derived) is mammalian cell invasion. These slender, highly motile organisms express a number of cell recognition and signaling molecules that mediate attachment and penetration of a wide variety of cell types (recently reviewed in Burleigh & Woolsey, 2002). Host cell invasion is co-incident with generation of the nascent parasitophorous vacuole which occurs either by a lysosome-mediated process involving early recruitment and targeted exocytosis of host cell lysosomes at the parasite attachment site (Tardieux *et al*. 1992) or by invagination of the host cell plasma membrane (Woolsey *et al*. 2003). Both routes of entry ultimately deliver trypomastigotes to the lysosomal compartment (Woolsey *et al*. 2003) where they reside for several hours before disruption of the vacuole occurs, releasing parasites into the cytoplasm (Andrews, 1994). Differentiation to amastigotes, the intracellular replicative form, is completed in the host cytoplasm and replication begins at $\sim$24 hours post infection (hpi), continuing every $\sim$12–16 hours for 4–5 days (Crane & Dvorak, 1979). In response to unknown cues, amastigote proliferation ceases at this point and differentiation to trypomastigotes ensues. Rupture of the infected cell releases trypomastigotes into the bloodstream, where they disseminate throughout the body, invade cells at distal sites, and can be ingested by the triatomine vector, thereby completing the insect–host transmission cycle. For the period constituting the acute stage of infection in the host (6–8 weeks) this replicative cycle continues and parasites are readily detectable in the blood and associated with inflammatory lesions present in many organs. *T. cruzi* growth in the acute stage is ultimately controlled by host immune mechanisms (Tarleton *et al*. 1996; Lima *et al*. 1997; Michailowsky *et al*. 2001; Duthie *et al*. 2002) and parasites are effectively cleared from the majority of tissues. However, parasite persistence at low levels in cardiac and smooth muscle contributes the eventual progression to cardiomyopathy and digestive disease in one-third of chronically infected individuals. The molecular basis for tissue tropism exhibited by this pathogen is unknown, but represents a fascinating aspect of *T. cruzi* biology and its complex interactions with the host.

IN VITRO VERSUS *IN VIVO T. CRUZI* INFECTION MODELS

It can be successfully argued that *in vitro* studies of *T. cruzi* infection are far removed from the range of complex responses occurring at a site of *T. cruzi* infection in vertebrate hosts or during progression to chronic Chagas' disease. Nevertheless, the nature of the question to be addressed will influence choice of an *in vitro* or *in vivo* infection model. If, for example, the goal is to identify host responses critical for intracellular growth and survival of the parasite it is likely that a transcriptional or cellular change reflecting this response may be restricted to the cell harbouring an intracellular parasite. If the uninfected cells in a population outnumber the infected cells (which would most certainly occur in an infected tissue sample) only robust changes occurring in infected cells or cellular responses to soluble factors secreted from infected or surrounding cells will be reliably detected. *In vitro* infection models permit manipulation of the multiplicity of infection (m.o.i) where on average of $\geqslant 1$ parasite/cell can be obtained, thereby facilitating detection of even modest changes in parasite-containing cells. Furthermore, host cell transcriptional responses arising from exposure of cells to soluble factors secreted from the infected monolayer (e.g. cytokines) can readily be distinguished from the response in an infected cell by employing a transwell cell culture system.

One of the therapeutic goals for treatment of Chagas' disease is to offset disease progression which occurs in only $\sim$30% of *T. cruzi*-infected individuals. A better understanding of the molecular events preceding or coinciding with disease onset may provide molecular markers for earlier prognosis as well as targets for preventing disease progression even in the absence of complete clearance of *T. cruzi* from tissues. Analysis of the range of transcriptional changes occurring in tissues extracted from *T. cruzi*-infected animals models of Chagas' disease will provide an entry point to further dissection of the complex response. Transcriptional responses occurring on a global level in infected tissue for example those triggered by secreted (e.g. cytokines) or diffusible (e.g. nitric oxide, oxygen radicals) factors may be distinguished from the more immediate responses taking place at a localized site of infection (inflammatory lesion) by coupling a selective approach such as laser dissection microscopy with cDNA microarray analysis (Ohyama *et al*. 2000; Matsui *et al*. 2003).

T. CRUZI-INDUCED TRANSCRIPTIONAL RESPONSES IN ISOLATED MAMMALIAN CELLS

At the most fundamental level, successful establishment of infection in the host is likely to depend on the ability of a pathogen to manipulate the host cell in favour of its survival and to subvert detrimental host defense mechanisms. The extent to which the parasite is able to derive essential nutrients and subvert detrimental host responses will clearly impact its ability to replicate in a mammalian cell. Specific

metabolic stresses on the host cell are likely to be compensated for by increased expression of the relevant host cell biosynthetic enzymes in the cell. This was recently demonstrated in *Toxoplasma gondii*-infected cells where host cell transcripts encoding several enzymes in the mevalonate pathway were upregulated in response to infection (Blader, Manger & Boothroyd, 2001). This transcriptional response correlated well with the knowledge that *T. gondii* cannot synthesize sterols via the mevalonate pathway and must scavenge them from the host cell (Coppens, Sinai & Joiner, 2000; Charron & Sibley, 2002). Thus, identification of global changes in host cell transcription in response to an intracellular pathogen is a valid approach to identifying novel pathways required for pathogen intracellular survival. Moreover, coupling this approach with an analysis of concurrent changes in parasite gene expression would provide a more powerful method to unravel the complex dialogue between parasite and host cell. The increasing availability of genomic tools for protozoan parasites and their hosts will facilitate such analyses.

Intracellular parasitism by *T. cruzi* indicates a requirement for amastigotes to derive essential components from the host cell (Davies, Ross & Gutteridge, 1983). Despite recent reports that *T. cruzi* evokes a pro-survival response in isolated primary cells under conditions of stress (Chuenkova *et al*. 2001; Heussler, Kuenzi & Rottenberg, 2001; Aoki *et al*. 2003), the cellular and molecular requirements for intracellular growth and survival of *T. cruzi* amastigotes in the host are poorly understood. To begin to elucidate *T. cruzi*-dependent responses that are essential for intracellular survival one can begin to catalogue the 'consensus' transcriptional response to *T. cruzi* in a variety of host cell types. Furthermore, host responses elicited specifically by *T. cruzi* can be determined by comparing the transcriptional profile in a given host cell type to those evoked by other intracellular pathogens. To date, there is only one study reporting of the use of DNA microarrays to examine *T. cruzi*-induced responses in mammalian host cells *in vitro* (de Avalos *et al*. 2002). This study was designed to facilitate comparison of *T. cruzi*-dependent changes in host cell gene expression at defined time points with that of *T. gondii* (Blader *et al*. 2001). Both studies, utilized human foreskin fibroblasts (HFF) for infection at similar time points and hybridizations were carried out using DNA microarrays from the same printings which contained $>27\,000$ human sequences. Furthermore, data were analyzed using similar criteria and methodology, providing an excellent opportunity to identify both common and pathogen-specific fibroblast responses to these two intracellular parasites. However, unlike comparable studies that identified a range of similarly modulated host cell transcripts in response to different bacterial pathogens (Nau *et al*. 2002), the overall transcriptional response to *T. cruzi* and *T. gondii* was strikingly dissimilar (Blader *et al*. 2001; de Avalos *et al*. 2002).

Most remarkable was the unexpected finding that no significant (i.e. greater than the arbitrary cut-off of 2-fold change) or reproducible induction of host cell gene expression was observed in response to *T. cruzi* at early time points of infection in HFF (2,4,6 hpi) (de Avalos *et al*. 2002). Thus despite considerable perturbation of the host cell at both the signaling and cellular levels during the invasion process (Burleigh & Woolsey, 2002), infected fibroblasts do not elicit a significant transcriptional response for several hours following infection (de Avalos *et al*. 2002). This contrasts markedly with the host cell response to *T. gondii* for which a rapid upregulation of HFF transcripts was observed (Blader *et al*. 2001). At 2 hpi ~ 60 known, unique genes were reproducibly upregulated and 15 genes were repressed in *T. gondii*-infected fibroblasts. The immediate/early response to *T. gondii* can be categorized as a host defense response with the upregulation of a set of immunomodulatory genes, many of which were shown to be triggered by soluble molecules secreted or released from *T. gondii* tachyzoites (Blader *et al*. 2001). A comparable response to soluble *T. cruzi* factors was not observed in HFF (de Avalos *et al*. 2002). Again, this was somewhat surprising given that infective *T. cruzi* trypomastigotes shed an abundance of surface glycoproteins (Goncalves *et al*. 1991; Kesper *et al*. 2000; Magdesian *et al*. 2001), secrete enzymes (Scharfstein *et al*. 2000; Grellier *et al*. 2001; Chamond *et al*. 2003) and other agonists (Burleigh *et al*. 1997) capable of activating a number of early signaling pathways in mammalian host cells (Burleigh *et al*. 1997; Scharfstein *et al*. 2000; Yoshida *et al*. 2000; Chuenkova *et al*. 2001). From this comparative analysis, it appears that *T. cruzi* and *T. gondii* have very different strategies for establishment of infection in the host. *T. gondii* immediately alerts the host immune system to its presence whereas *T. cruzi* may have more of a stealth approach to early infection. However, until additional experiments are conducted using a wider range of host cell types, these generalizations are put forward with caution.

Whilst no significant induction of fibroblast genes was observed early in the *T. cruzi* infective process, repression of 6 fibroblast transcripts was consistently observed (de Avalos *et al*. 2002). Three of these transcripts encode cysteine-rich, secreted extracellular matrix proteins with roles in fibrosis (connective tissue growth factor, CTGF), angiogenesis (cysteine-rich angiongenic factor, Cyr61) and tissue remodeling (a disintegrin and metalloprotease with a thrombospondin domain protein, ADAM-TS1). Of the early repressed genes, only CTGF was found to be repressed at later time points of infection ($\geqslant 24$ h)

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and was the only transcript in the entire analysis found to be modulated by secreted/released molecules from infective *T. cruzi* trypomastigotes (de Avalos *et al*. 2002). CTGF plays a central role in the TGFβ-regulated fibrogenic and wound repair response in tissue (Grotendorst, 1997; Duncan *et al*. 1999). In fibrotic disorders, CTGF and downstream fibrogenic genes such as collagens and fibronectins are aberrantly upregulated (Igarashi *et al*. 1996) and CTGF has been considered a potential therapeutic target for the treatment of fibrosis (Blom, Goldschmeding & Leask, 2002; Simms & Korn, 2002). For this reason, it is intriguing that *T. cruzi* produces a soluble molecule capable of rapidly downregulating CTGF expression without affecting TGFβ mRNA levels in dermal fibroblasts (de Avalos *et al*. 2002). Identification of a parasite-encoded CTGF repressive activity may have important consequences for the further study and/or treatment of fibrotic disorders. Moreover, investigation of the host fibrogenic pathway in the context of animal models of *T. cruzi* infection may reveal a role for the immediate/early and sustained repression of CTGF in the establishment of infection in the host.

Following the marked delay in the onset of host cell transcriptional changes in response to *T. cruzi*, a consistent upregulation of ~100 HFF genes was observed at 24 hpi and 6 transcripts were downregulated (de Avalos *et al*. 2002). The majority of transcripts induced in response to *T. cruzi* infection were interferon-stimulated genes (ISGs). This response is triggered by the production of type I interferon (IFNβ) from infected HFF, detectable by ~18 hpi (de Avalos *et al*. 2002). In addition to their well-characterized roles as anti-viral cytokines, type I interferons have a range of immunomodulatory activities including activation of natural killer cells (Biron *et al*. 1999) and inhibition of IL-12 responsiveness and IFNγ production in NK cells (Nguyen & Benveniste, 2000). It remains to be determined if and how type I interferons influence the early establishment of *T. cruzi* infection in the host.

HOST RESPONSE TO *T. CRUZI* INFECTION *IN VIVO*

In two recent independent studies, DNA microarray approaches were used to examine the global transcriptional responses in *T. cruzi*-infected heart tissue (Garg *et al*. 2003; Mukherjee *et al*. 2003). Using well-established mouse models of chronic chagasic cardiomyopathy both studies examined changes in host cardiac gene expression associated with progression to disease at ~100 days post infection. Both groups presented data clearly demonstrating myocarditis, tissue damage or dilated hypertrophy and reported upregulation of atrial natriuretic peptide precursor in their respective chronic Chagas' disease models (Garg *et al*. 2003; Mukherjee *et al*. 2003) a strong indicator of cardiac pathogenesis (Vikstrom *et al*. 1998). In spite of these similarities, the overall profile of cardiac gene expression presented in the two papers, revealed few common features, possibly owing to differences in experimental design, such as *T. cruzi* infection model and array hybridization methods (Garg *et al*. 2003; Mukherjee *et al*. 2003).

Transcriptional profiling in infected heart tissue at different stages of the *T. cruzi* infective process, 3, 37 and 110 days post infection which corresponds to immediate/early, acute and chronic infection respectively led to several general observations (Garg *et al*. 2003). The immediate/early response to *T. cruzi* infection in cardiac tissue consisted mainly of induction of inflammatory mediators, cytokines, chemokines and interferon-stimulated genes. Overall these responses were more pronounced at 37 days post-infection, correlating with the peak of acute phase infection where parasitaemia was relatively high and parasites were abundant in tissues. In addition to increased expression of immunomodulatory genes, significant upregulation of several pro-fibrogenic and hypertrophic genes was observed during the acute stage of infection, which likely reflects wound repair processes activated in response to tissue damage. Following the onset of chronic disease in these animals, a general repression of the transcriptional response was observed with many of the inflammatory, extracellular matrix and hypertrophic genes previously upregulated, now muted (Garg *et al*. 2003). Decreased expression of genes encoding key cytoskeletal elements, such as cardiac troponins, is suggestive of disruption of the sarcomeric filament system in the heart. Transmission electron microscopy of sections through diseased hearts at 110 days post infection reveal regions of extensive cardiac remodeling and provide evidence for sarcomeric disruption (Garg *et al*. 2003). In contrast, data from Mukherjee *et al*. (Mukherjee *et al*. 2003) indicate that ECM genes, especially those associated with fibrosis, e.g. procollagen type I, αI, were upregulated in this chronic disease model, similar to that observed at day 37 post infection (Garg *et al*. 2003). The use of different *T. cruzi* infection models (Brazil strain and C57BL/6x129sv mice; Mukherjee *et al*. 2003) versus (Silvio X10/4 strain and C3H/HeN mice; Garg *et al*. 2003) might produce differences in the kinetics of onset of severe disease leading to inconsistent findings.

One of the most significant observations made was the generalized depression of mitochondrial function during progression to chronic disease (Garg *et al*. 2003). A dramatic downregulation of transcripts encoding several components of the mitochondrial oxidative phosphorylation pathway, specifically NADH-ubiquinone oxidoreductase and cytochrome c oxidase, suggests that mitochondrial function is severely compromised in *T. cruzi*-infected hearts. Early indications of this are evident at 37 days post-infection where transcripts for cytochrome c oxidase

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subunits are lower than in control mice, however, a more dramatic repression of multiple components of the oxidative phosphorylation pathway is observed at later stages of infection (Garg *et al*. 2003). Supportive evidence for mitochondrial dysfunction was gained by RT-PCR and western blot analysis as well as measurements of the activity of key enzymes in this pathway in control and infected hearts. Furthermore, morphological abnormalities in mitochondrial structure and distribution in cardiac myocytes were readily observed, in which mitochondrial swelling and lipid accumulation was evident. Thus, despite the fact that this study was limited by the number of genes analyzed (1176 cDNAs) the findings present the basis for an emerging picture of distinct molecular events occurring in the *T. cruzi*-infected heart during acute infection and progression to chronic disease (Garg *et al*. 2003).

T. CRUZI DIFFERENTIATION

In both the invertebrate and mammalian hosts, *T. cruzi* undergoes distinct developmental changes: epimastigote to metacyclic differentiation in the insect vector and the trypomastigote-amastigote cycle in the vertebrate host. In the first published study of its type, Minning and colleagues analyzed changes in parasite transcript abundance early in the trypomastigote to amastigote differentiation process *in vitro* using DNA microarrays containing 4400 *T. cruzi* sequences (Minning *et al*. 2003). After shifting trypomastigotes to acidified medium for 2 hours to initiate transformation to amastigotes (mimicking the low pH conditions of the *T. cruzi* vacuole) RNA was harvested from untreated and treated parasites, and fluorescently labeled (Cy3 and Cy5) cDNA probes generated and hybridized to the arrays. In this analysis, 38 unique genes were repeatedly upregulated in the differentiating parasites and 11 transcripts were repressed (Minning *et al*. 2003). Several of the transcripts expressed in higher abundance in trypomastigotes were members of the trans-sialidase gene family. Trans-sialidases and the related catalytically inactive gp85 family are surface expressed glycosylphosphatidylinositol-linked surface proteins. Some members exhibit stage-specific expression (Santos, Garg & Tarleton, 1997) and while the full range of functions of this family of proteins has not been elucidated, it is clear that trans-sialidase/gp85 proteins expressed on trypomastigotes facilitate parasite invasion of the mammalian cells. Using a specific monoclonal antibody to trypomastigote trans-sialidase molecules it was previously demonstrated that this surface protein is rapidly shed from trypomastigotes following host cell invasion and not expressed again until amastigote to trypomastigote transformation occurs after several days of infection (Frevert, Schenkman & Nussenzweig, 1992). Thus, a rapid decrease in transcript abundance for several members of this gene family (Minning *et al*. 2003) is in agreement with previous observations of the biology of the early *T. cruzi*-host cell interaction.

Given that regulation of gene expression in *T. cruzi* is thought to occur primarily at the post-transcriptional level many differentially expressed genes may be missed using an approach that relies on relative transcript abundance such as DNA microarray analysis. There are several examples of this that have been documented during metacyclogenesis, the epimastigote to metacyclic transformation. Metacyclogenin is a 13 kDa protein that is transiently expressed during the epimastigote to metacyclic differentiation process but not in either epimastigote or metacyclic trypanosomes (Avila *et al*. 2001). Northern blot analysis demonstrated the presence of metacyclogenin mRNA in total RNA extracted from both replicating and differentiating epimastigotes. However, the transcript was specifically mobilized to the polysomes and translated only in differentiating epimastigotes (Avila *et al*. 2001). Similar findings have been reported for several genes that are differentially expressed during metacyclogenesis including topoisomerase II (Fragoso *et al*. 1998) and Tclmp4 (Fragoso *et al*. 2003). Thus selective mobilization of at least some mRNA transcripts to polysomes for translation represents an important mechanism for regulation of stage-specific gene expression in *T. cruzi*. For additional detail the reader is referred to a recent review (Avila *et al*. 2003).

PERSPECTIVES

The pilot studies outlined in this review most certainly represent the first of many future investigations that will employ relatively high-throughput genomic and proteomic approaches to characterize *in vitro* and *in vivo* models of *T. cruzi* infection as well as the parasite developmental processes. Novel and potentially interesting host responses to *T. cruzi* infection were reported in the studies under consideration here; however, it is too early to determine whether these represent universal and biologically relevant responses or whether some of the responses are specific to a given model system. An indication that differences in experimental design could eventually contribute to significant confusion in the field is suggested when comparing results from the *in vivo* studies (Garg *et al*. 2003; Mukherjee *et al*. 2003), which do not correlate. With spotted gene arrays becoming increasingly accessible tools to study differential gene expression, we can expect an abundance of large data-sets relatively soon. Since gene expression data are only as useful as they can be reliably compared with other data-sets generated in the same or different laboratories, the challenge lies

in generating data-sets that will be useful to the entire community where data accessibility and a level of standardization in microarray data reporting will be key components (Brazma, 2001; Brazma *et al*. 2001; Ball *et al*. 2002). As pointed out in a recent review on the uses and limitations of microarray technology for parasitologists, Boothroyd and colleagues stress this point eloquently with a discussion of the use of reference samples that facilitate comparisons of array data across different experiments (Boothroyd *et al*. 2003) and refer the reader to a website that presents guidelines for a more standardized approach to conducting analyzing and reporting data from microarray experiments (http://www.mged.org).

We are embarking on an era in which genomics, proteomics and bioinformatics provide a new and powerful set of tools to approach a number of fundamental questions in *T. cruzi* biology. These tools will greatly facilitate genomic fingerprinting of field isolates, comparisons of virulent versus attenuated *T. cruzi* strains (Weston, Patel & Van Voorhis, 1999; Basombrio *et al*. 2000), and drug-resistant and -sensitive parasites (Nozaki, Engel & Dvorak, 1996; Engel *et al*. 2000). Standardization of experimental protocols and methods of data reporting will facilitate comparisons of host responses elicited by *T. cruzi* and other important pathogens under a given set of conditions. Together, the availability of these technologies affords opportunity and optimism for the future identification of novel drug and vaccine targets for the treatment and prevention of Chagas' disease.

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